Plasma miR-1290 Is a Novel and Specific Biomarker for Early Diagnosis of Necrotizing Enterocolitis—Biomarker Discovery with Prospective Cohort Evaluation

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Objective To discover specific circulating microRNA (miRNA) biomarkers for the early differentiation of necrotizing enterocolitis (NEC) from neonatal sepsis and inflammatory conditions.

Study design The study comprised 3 distinct phases: differential microarray analysis to compare plasma miRNA expression profiles of NEC vs sepsis and non-NEC/nonsepsis cases, a case-control study to quantify dysregulated miRNAs as potential specific biomarkers of NEC, and a prospective cohort study to assess the diagnostic usefulness of the best miRNA biomarker(s).

Results A distinct miRNA expression profile was observed in the NEC compared with the sepsis and non-NEC/nonsepsis groups. miR-1290, miR-1246, and miR-375 were discovered to be specific biomarkers of NEC in the case-control study. In the cohort study (n = 301), plasma miR-1290 (day 0; >220 copies/μL) provided the greatest diagnostic usefulness for identifying both mild medical and severe surgical NEC cases. Of 20 infants with miR-1290 >650 copies/μL, 15 were diagnosed with NEC. Incorporating C-reactive protein (day 1; >15.8 mg/L) for cases with intermediate levels (220-650 copies/μL) in a 2-stage algorithm further optimized the diagnostic profile with a sensitivity of 0.83, a specificity of 0.96, a positive predictive value of 0.75, and a negative predictive value of 0.98. Importantly, 7 of 36 infants with NEC (19.4%) could be diagnosed 7.8-32.2 hours earlier (median, 13.3 hours) using miR-1290.

Conclusions Plasma miR-1290 is a novel and specific biomarker that can effectively differentiate NEC cases from neonatal sepsis. miR-1290 facilitates neonatologists to confidently and timely reach a decision for early transfer of sick infants with NEC from community-based hospitals to tertiary surgical centers. *(J Pediatr 2019;205:83-90).*

Necrotizing enterocolitis (NEC) is a severe inflammatory gastrointestinal (GI) condition that predominantly affects preterm infants.[1] A recent survey reported that, although infant deaths associated with prematurity, infection, central nervous system injuries, and respiratory diseases have decreased, NEC-associated deaths were paradoxically increased.[2] Those who survive often suffer severe morbidities.[1,3] The early diagnosis of NEC is difficult because the initial clinical features are often nonspecific and difficult to differentiate from other neonatal conditions, including functional GI dysmotility of prematurity[4] or sepsis-induced ileus. Nonspecific biomarkers, such as acute phase proteins,[5-8] cell-surface antigens,[9-11] cytokines, and chemokines[12,13] are unable to differentiate between NEC and neonatal sepsis. Clinically, it is important to differentiate the 2 conditions, because patients with NEC are offered a substantially different treatment protocol, including serial abdominal radiographs, prolonged total parenteral nutrition, and broader antibiotic coverage for bowel organisms. Thus, the management of infants with suspected NEC could be confidently rationalized at an earlier stage and would facilitate efficient transfer of sick infants with NEC from community-based hospitals to tertiary surgical centers. To date, there have been no effective and specific plasma biomarkers for early diagnosis of NEC, especially to distinguish between mild medical NEC cases from neonatal sepsis.[14,15]

MicroRNAs (miRNA18-24 nucleotide noncoding RNAs) are chemically stable in biofluid mediums such as blood and stool.[16] Specific miRNAs have been implicated in ileal and colonic mucosa, and blood specimens of patients with inflammatory bowel disease,[17,18] suggesting that these markers could act as potential diagnostic indicators for GI injury.[18-20] In our recent studies on genome-wide expression arrays of tissues and blood, we identified dysregulated messenger RNAs

BPD	Bronchopulmonary dysplasia	NEC	Necrotizing enterocolitis
CRP	C-reactive protein	NPV	Negative predictive value
DOR	Diagnostic OR	PPV	Positive predictive value
GI	Gastrointestinal	ROC	Receiver operating characteristic
LHR-	Negative likelihood ratio	RT-qPCR	Reverse transcription quantitative polymerase chain reaction
LHR+	Positive likelihood ratio		

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Supported by the donation from the charity organization Providence Foundation Limited (Project No. 6902896). The funding organization did not play any role in the study design, the collection, analysis and interpretation of data, the writing of the report and the decision to submit the paper for publication. The authors declare no conflicts of interest.

https://doi.org10.1016/j.jpeds.2018.09.031

and miRNAs in NEC compared with spontaneous intestinal perforation and patients with septicemia.[21,22] These findings prompted us to hypothesize that some of these miRNAs are specifically present in the bowel mucosa and could spill into the circulation at an early stage of gut inflammation, and thus, could act as early and specific biomarkers for NEC.

The objectives were to identify dysregulated miRNAs in infants with NEC at clinical presentation, using a hypothesis-free approach by microarray analysis of plasma miRNAs from: NEC, gram-negative septicemia, gram-positive septicemia, and non-NEC/nonsepsis infants; to validate potential miRNA biomarkers for the specific diagnosis of NEC in a case-control study; and in a prospective cohort study to evaluate the best biomarkers by establishing the optimal cutoff values and diagnostic usefulness of 1 or combination of target miRNAs for accurate differentiation of NEC from neonatal sepsis and other neonatal inflammatory conditions, for example, bronchopulmonary dysplasia (BPD).

Methods

This study was conducted in a university-affiliated tertiary neonatal center. Details of patient recruitment criteria, sepsis screening protocol, and stringent classification of NEC and neonatal sepsis have been described previously.[6,23] Our sample size calculation suggested that 298 suspected sepsis/NEC episodes would be required for the study (**Appendix 1**; available at www.jpeds.com). There were 301 suspected sepsis/NEC episodes from 232 preterm infants with signs and symptoms suspected of late-onset sepsis (>72 hour of postnatal age) or NEC and requiring full sepsis evaluation with antimicrobial treatment that were consecutively recruited into the study over a period of 54 months. A standard full sepsis screening was performed (day 0) and blood for miRNA biomarkers was simultaneously collected. In the initial 7 proven NEC cases, miRNA concentrations in paired day 0 and day 1 (24 hours later) samples were compared. Each episode was classified into the following distinct groups: group 1, proven NEC; group 2a, culture-proven gram-negative sepsis; group 2b, culture-proven gram-positive sepsis; group 2c, clinical sepsis; group 3, non-NEC/nonsepsis; and group 4, controls.

Group 1: Proven NEC

Group 1 included cases with the modified Bell's classification of equal or greater than stage II. All cases were confirmed by 2 experienced neonatologists and a senior pediatric radiologist. Patient identity and biomarkers results were completely blinded to the assessors. All infants requiring surgery had specimens routinely sent for histologic examination to differentiate NEC from spontaneous intestinal perforation or other intestinal pathologies (eg, congenital absence of intestinal muscle).

Group 2a: Culture-Proven Gram-Negative Sepsis

Group 2a included cases with gram-negative septicemia or gram-negative organisms recovered from the cerebrospinal fluid or other body fluids, including peritoneal fluid or pus from abscesses.

Group 2b: Culture-Proven Gram-Positive Sepsis

Group 2b included cases with gram-positive septicemia or gram-positive organisms recovered from the cerebrospinal fluid or other body fluids.

Group 2c: Clinical Sepsis

Group 2c included cases with >3 signs and symptoms of sepsis, plus elevated serial C-reactive protein (CRP) levels (>10 mg/L) and who were given a full course (>7days) of parenteral antibiotics resulting in continuous clinical improvement and decreasing levels of CRP. However, no pathogens were isolated from their laboratory specimens.

Group 3: Non-NEC/Nonsepsis

Infants who met the initial criteria for sepsis screening but subsequently diagnosed to have other conditions unrelated to sepsis or NEC, such as exacerbation of BPD, apnea of prematurity, severe anemia, heart failure, fluctuation of body temperature secondary to environmental factors, or functional GI dysmotility of prematurity were included in group 3. Groups 2a, 2b, 2c, and 3 infants did not have specific clinical, histologic, or radiologic features of NEC, and together they constituted the non-NEC groups.

Group 4: Controls

Residual EDTA blood was used for miRNA measurement in 20 healthy (ie, infants not subjected to sepsis workup) preterm infants during one of their routine weekly blood samplings for complete blood counts and liver/renal function tests. Twelve samples were obtained at a relatively early age of 1-3 weeks of age and 8 were obtained late at 4-6 weeks of age, so as to assess the impact of postnatal age on miRNA levels.

Study Design

The study was conducted in 3 distinct phases as illustrated in **Figure 1** (available at www.jpeds.com).

Phase 1: miRNA Microarray Analyses

Differential miRNA microarray analyses of plasma specimens obtained at the time of sepsis evaluation (day 0) from proven NEC, gram-negative septicemia, gram-positive septicemia, and non-NEC/nonsepsis (n = 4 per group), were performed. Potential miRNA biomarkers were selected from the criteria of a >2-fold elevation and with a significant difference between NEC and non-NEC cases ($P < .05$).

Phase 2: Case-Control Study

Based on results of the phase 1 study, 7 potential miRNA biomarkers were selected and measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR). A case-control study was then performed to evaluate their number of copies in plasma specimens of NEC (5 proven medical NEC plus 5 surgical NEC cases), septicemia (gram negative and gram positive, n = 10), and non-NEC/nonsepsis infants (n = 10). An additional group of control infants (n = 20) was included and their specimens were obtained between 1 and 6 weeks of postnatal age, representing the most frequent timing of occurrence of NEC (n = 12 [1-3 weeks]; n = 8 [4-6 weeks]).

Phase 3: Prospective Cohort Study

A cohort of consecutively recruited episodes of NEC (n = 36) vs non-NEC (ie, gram-negative sepsis, n = 19; gram-positive sepsis, n = 33; clinical sepsis, n = 49; and non-NEC/nonsepsis, n = 164; total N = 265 cases) was used to assess the 3 most effective candidate miRNA biomarkers derived from the case-control study. The optimal cutoff values and diagnostic usefulness of the most clinically useful single or combination of biomarkers for early diagnosis of NEC were determined.

Laboratory Analyses

Details of plasma sample preparation, microarray and RT-qPCR measurement of miRNAs were described in **Appendix 2** (available at www.jpeds.com).

Statistical Analyses

The χ^2, Fisher exact, Mann-Whitney *U*, and Kruskal-Wallis tests were used to compare the demographic data, clinical characteristics, and biomarker concentrations among groups, where appropriate. A receiver operating characteristic (ROC) curve was constructed for each biomarker and the optimal cutoff value calculated by minimizing the number of misclassified episodes. The optimal cutoff values were selected with relatively high sensitivity and specificity approaching 85%. The diagnostic usefulness in terms of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LHR+), negative likelihood ratio (LHR-), and diagnostic OR (DOR) were determined. Spearman correlation was performed between miR-1290 levels of NEC or control infants and postnatal age or gestation weeks. All statistical analyses were performed using SPSS 21.0 (IBM, Armonk, New York).

Ethical Approval

The study was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee. Written consent was obtained from parents of all infants.

Results

miRNA Microarray Profiles of Plasma Specimens

Analysis of plasma from the confirmed NEC, gram-negative septicemia, gram-positive septicemia, and non-NEC/nonsepsis groups revealed distinct clustering of gene expressions in the NEC compared with other groups (**Figure 2**; available at www.jpeds.com). However, there was some overlap between gram-positive septicemia and non-NEC/nonsepsis miRNA expression clusters. NEC demonstrated significant upregulation and downregulation of 230 miRNAs (2.0- to 26.34-fold) and 16 miRNAs (0.50- to 0.16-fold) compared with the non-NEC groups, respectively. Based on the criteria of (i) a >2-fold elevation and (ii) significantly increased levels in NEC specimens compared with other groups, 7 miRNAs (miR-1290, miR-1246, miR-1233-5p, miR-619-5p, miR-1469, miR-375, and miR-665) were selected for further investigation (**Table I**; available at www.jpeds.com).

Case-Control Study to Identify Potential Plasma miRNAs for the Early Diagnosis of NEC

The clinical characteristics of case-control infants were summarized in **Table II** (available at www.jpeds.com). Seven selected miRNAs from microarray analysis were further analyzed by RT-qPCR. Levels of 4 miRNAs (miR-1290, miR-1246, miR-375, and miR-619-5p) were within detectable ranges. Significantly increased copies of miR-1290 (median, 6.78-fold), miR-1246 (7.62-fold), and miR-375 (6.52-fold) were observed in the NEC group compared with the septicemia group (**Figure 3**). miR-1290 (17.34-fold and 8.61-fold), miR-1246 (7.39-fold and 2.12-fold), and miR-375 (5.33-fold and 7.72-fold) were also significantly higher in the NEC group compared with non-NEC/nonsepsis and control groups, respectively (**Figure 3**). In contrast, miR-619-5p was not significantly different between the NEC and septicemia groups ($P = .739$). In an independent assessment, we demonstrated a significant decrease in levels of miR-1290, miR-1246, and miR-375 at day 1 compared with day 0 in proven NEC cases (n = 7, $P < .05$; **Figure 4** [available at www.jpeds.com]), indicative of their characteristics as early diagnostic biomarkers. Thus, miR-1290, miR-1246, and miR-375 were selected for further evaluation in the prospective cohort study.

Prospective Cohort Study to Determine the Diagnostic Usefulness of Novel miRNA Biomarkers

Clinical Characteristics. The clinical characteristics of NEC and non-NEC groups (gram-negative sepsis, gram-positive sepsis, clinical sepsis, and non-NEC/nonsepsis groups) are summarized in **Table III** (available at www.jpeds.com). There were no significant differences in gestational age, birthweight, sex, Apgar scores at 1 and 5 minutes, or duration of hospitalization between the NEC and non-NEC groups. As expected, owing to differences in the nature of diseases in various groups, the postnatal age at onset of illness and number of disseminated intravascular coagulation cases and deaths were significantly different between NEC and some non-NEC groups.

ROC Curve. ROC curves of miR-1290, miR-1246, and miR-375 were constructed (**Figure 5**; available at www.jpeds.com), and their areas under the curves for diagnosis of NEC were 0.92, 0.84, and 0.87, respectively ($P < .001$).

Diagnostic strategy for identifying NEC cases. The number of miRNA copies/μL of miR-1290, miR-1246, and miR-375 was significantly higher in the NEC group compared with those in gram-negative sepsis, gram-positive sepsis, clinical sepsis, or non-NEC/nonsepsis groups individually ($P < .001$; **Table IV**). Thus, all 3 selected miRNAs were regulated in a similar manner and were specific biomarkers for NEC. In contrast, CRP was unable to differentiate NEC cases from gram-negative or gram-positive sepsis cases ($P > .05$; **Table IV**).

The optimal cutoff levels of miR-1290, miR-1246, and miR-375 as computed from ROC curves, and their respective diagnostic usefulness are summarized in **Table V**. Plasma miR-1290 at the time of clinical presentation (day 0) with a cutoff

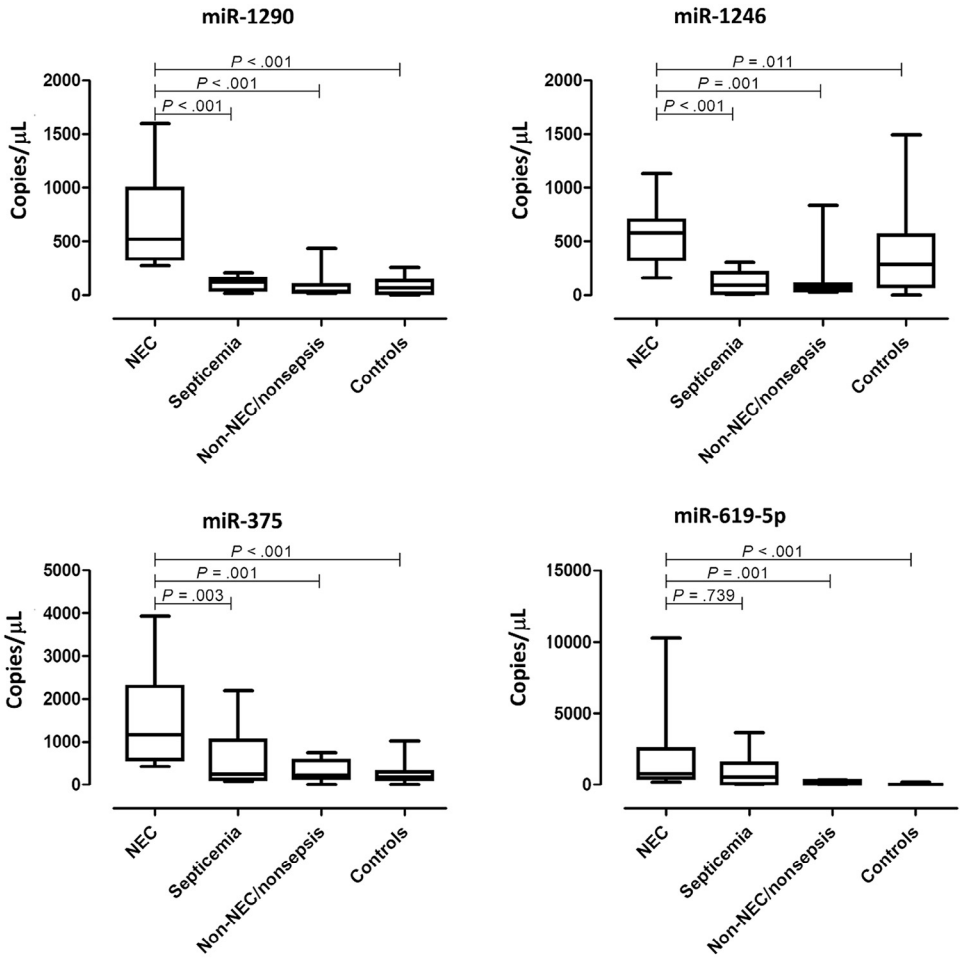

Figure 3. Expression of selected miRNAs in the case-control study. q-PCR validation of miRNAs in plasma samples of NEC (n = 10), septicemia (n = 10), non-NEC/nonsepsis (n = 10), and healthy control infants (n = 20). Results are median (IQR).

of >220 copies/µL provided the best diagnostic usefulness (sensitivity of 0.83, specificity of 0.92, PPV of 0.60, and NPV of 0.98) for early identification of both mild medical (19/23) and severe surgical (11/13) NEC cases (**Table V**). The LHR+ and LHR- were 11.04 and 0.18, respectively. In general, a test with an LHR+ value of >5 and an LHR- value of <0.2 would be considered clinically useful.[24] The results also indicated that cases with a plasma miR-1290 of > 650 copies/µL had a very high specificity (0.98), and good PPV (0.75). Thus, marker levels between 220 and 650 copes/µL may be considered an intermediate zone, because some false-positive NEC cases (n = 20) were included. By applying an additional criterion of a CRP of >15.8 mg/L on day 1, the overall specificity and PPV were improved from 0.92 to 0.96, and 0.60 to 0.75, respectively, without affecting the sensitivity and NPV (**Table V**). Using miR-1290 at the time of sepsis workup (day 0) in cases with levels ranging from 220 to 650 copies/µL and screening with CRP 24 hours later (**Figure 6**). Applying this simple 2-stage algorithm, only 10 of 265 non-NEC cases (3.8%) were mistakenly classified as NEC, and 6 of 36 NEC cases (16.7%) escaped detection. This strategy further enhanced the diagnostic usefulness with a sensitivity of 0.83, a specificity of 0.96, a PPV of 0.75, an NPV of 0.98, an LHR+ of 22.08, an LHR- of 0.17, and a DOR of 127.50. This 2-stage algorithm and the resulting diagnostic outcomes are summarized in **Figure 6**.

We also examined the time difference between confirmation of NEC using definitive clinical/radiologic features and miR-1290 results. Of 36 NEC cases, 25 (69%) had definitive radiologic signs of NEC at the time of sepsis workup. Of the remaining 11 delayed cases (31%), 7 (19%; 2 surgical and 5 medical NEC) could benefit from the miR-1290 test for an earlier diagnosis by 7.8 to 32.2 hours (median, 13.3 hours). The other 4 cases (1 surgical and 3 medical) had miR-1290 levels of <220 copies/µL.

There was no significant correlation between miR-1290 and gestational age or postnatal age in the NEC ($r = -0.075$, $P = .668$ and $r = -0.091$, $P = .598$) and healthy control groups ($r = -0.201$, $P = .395$ and $r = -0.066$, $P = .783$), respectively. Similarly, no significant correlation was observed between miR-1246 or miR-375 and gestational or postnatal age in either the NEC or control

Table IV. Expression levels of target plasma miRNAs and CRP concentrations of the NEC vs gram-negative sepsis, gram-positive sepsis, clinical sepsis, and non-NEC/nonsepsis groups

				Non-NEC groups				*P* values			
	NEC (group 1)	Combined non-NEC* (group 2(a, b, c) +3)	*P* values NEC vs non-NEC	Gram-negative sepsis (group 2a)	Gram-positive sepsis (group 2b)	Clinical sepsis (group 2c)	Non-NEC/ nonsepsis (group 3)	NEC vs gram-negative sepsis	NEC vs gram-positive sepsis	NEC vs clinical sepsis	NEC vs non-NEC/ nonsepsis
No. of episodes	36	265	—	19	33	49	164	—	—	—	—
miR-1290, copies/μL	520 (239-1093)	43 (14-92)	<.001	109 (28-174)	52 (28-92)	27 (11-59)	42 (12-88)	<.001	<.001	<.001	<.001
miR-1246, copies/μL	578 (101-814)	52 (16-108)	<.001	126 (10-266)	69 (31-149)	39 (16-81)	50 (13-103)	.001	<.001	<0001	<.001
miR-375, copies/μL	1018 (476-2196)	131 (40-279)	<.001	241 (43-487)	194 (142-365)	102 (36-216)	118 (33-261)	<.001	<.001	<.001	<.001
CRP mg/L (day 1)	57.4 (33.8-136.8)	5.1 (0.6-36.7)	<.001	91.5 (60.7-162.0)	53.1 (31.6-80.8)	28.2 (16.3-52.9)	0.6 (0.6-3.7)	.061	.334	<.001	<.001

Results are expressed as median (IQR).
*The non-NEC group comprised groups 2a, 2b, 2c, and 3.

Table V. Diagnostic usefulness of target miRNA biomarkers

Biomarkers	Cutoffs	Sensitivity	Specificity	PPV	NPV	LHR+	LHR-	DOR
miR-1290, copies/μL	220	0.83 (0.71-0.96)	0.92 (0.89-0.96)	0.60 (0.46-0.74)	0.98 (0.96-0.99)	11.04 (7.07-17.25)	0.18 (0.09-0.37)	61.25 (22.81-164.50)
	650	0.42 (0.26-0.58)	0.98 (0.96-1.00)	0.75 (0.56-0.94)	0.93 (0.89-0.96)	22.08 (8.54-57.12)	0.59 (0.45-0.78)	37.14 (12.30-112.20)
miR-1246, copies/μL	330	0.72 (0.58-0.87)	0.94 (0.92-0.97)	0.63 (0.49-0.78)	0.96 (0.94-0.98)	12.76 (7.50-21.71)	0.29 (0.17-0.50)	43.33 (17.68-106.19)
miR-375, copies/μL	422	0.81 (0.68-0.93)	0.85 (0.81-0.89)	0.42 (0.30-0.54)	0.97 (0.95-0.99)	5.34 (3.85-7.41)	0.23 (0.12-0.45)	23.30 (9.56-56.82)
CRP mg/L (day 1)	10	0.92 (0.83-1.01)	0.55 (0.49-0.61)	0.22 (0.15-0.28)	0.98 (0.96-1.00)	2.04 (1.73-2.41)	0.15 (0.05-0.45)	13.50 (4.04-45.10)
miR-1290 copies/μL (day 0) and CRP mg/L (day 1)	>650 **OR** >220 to 650 and CRP >15.8	0.83 (0.71-0.96)	0.96 (0.94-0.99)	0.75 (0.62-0.88)	0.98 (0.96-1.00)	22.08 (11.82-41.27)	0.17 (0.08-0.36)	127.50 (43.28-375.65)

CRP was taken 24 hours (day 1) after the initial clinical presentation of NEC.
Bracket values are 95% CIs.

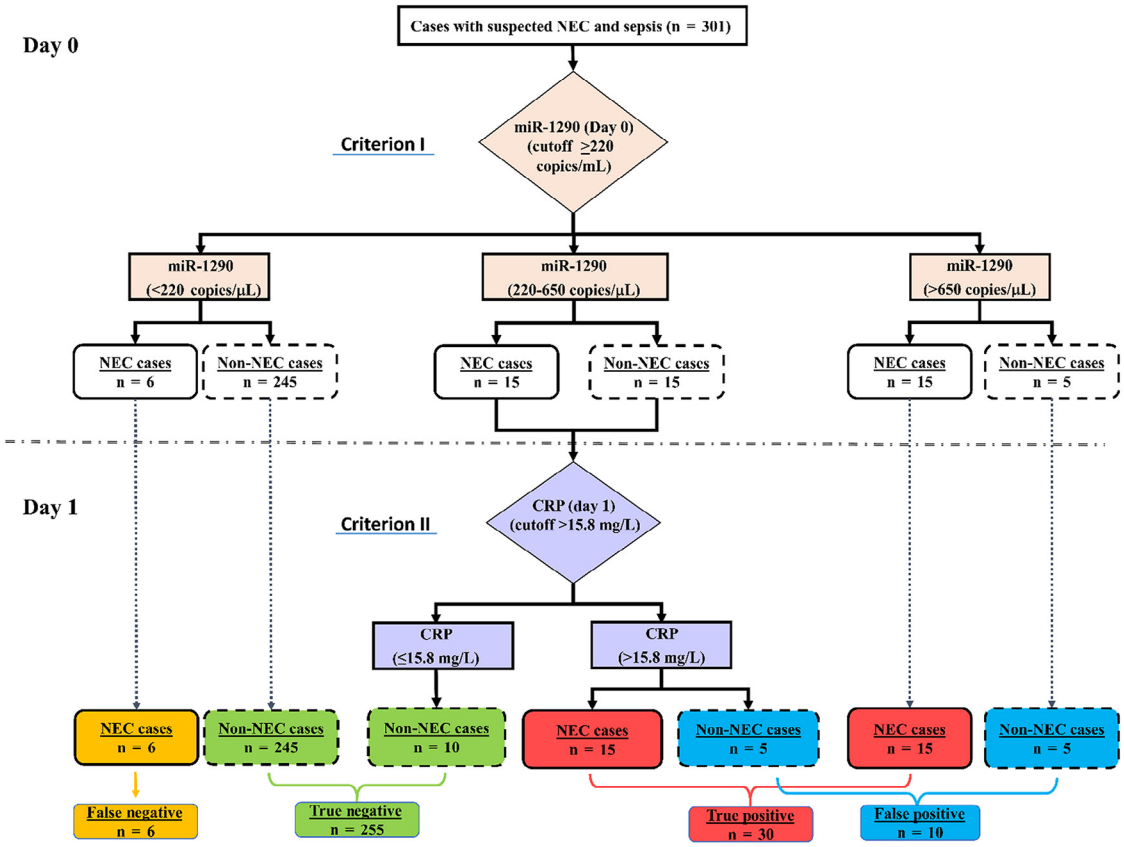

Figure 6. A flow diagram of the 2-stage algorithm using miR-1290 (day 0) and CRP (day 1) for identifying NEC cases.

groups, as illustrated in **Figures 7** and **Figure 8** (available at www.jpeds.com).

Discussion

This study provides a comprehensive evaluation of plasma miRNAs as specific biomarkers for the early diagnosis of NEC. We initiated our investigation using a hypothesis-free approach of global miRNA expression profiling on proven cases of NEC vs defined groups of non-NEC cases. Our data provide robust evidence that miR-1290 is a novel and specific biomarker that can effectively differentiate both medical (mild) and surgical (severe) NEC from neonatal sepsis or neonatal inflammatory conditions such as BPD with favorable diagnostic accuracy. We have also developed a simple 2-stage algorithm of incorporating plasma miR-1290 at the time of disease presentation (day 0) and CRP 24 hours later (day 1) to optimize accurate classification. The overall diagnostic usefulness reached a sensitivity of 0.83, specificity of 0.96, PPV of 0.75, NPV of 0.98, LHR+ of 22.08, LHR- of 0.17, and DOR 127.50, exceeding conventional recommendations for clinically useful biomarkers. Most current circulating protein biomarkers of NEC, including liver fatty acid binding protein, intestinal fatty acid binding protein, trefoil factor-3, or the L-FABP, I-FABP, trefoil factor 3 (LIT) score were only useful for identifying severe surgical NEC cases, but not early mild medical cases.[23] The combined use of miR-1290 and CRP enables neonatologists to promptly identify even mild NEC cases, adding confidence to the initiation of a specific NEC treatment protocol by stopping enteral milk feeding, initiating total parenteral nutrition early, introducing a wider spectrum of antibiotic coverage for gut and anaerobic organisms, and vigilant radiologic monitoring of intra-abdominal complications. In addition, miR-1290 can be especially useful in community-based hospitals that require efficient and timely transfer of sick infants with NEC to tertiary surgical centers.

To date, limited information is available concerning the involvement of miRNAs in the pathophysiology of NEC or their usefulness as diagnostic biomarkers. In our recent study, we reported that miR-1290 and miR-375 were dysregulated in intestinal tissues of infants with NEC, and could be involved in THSB1-mediated angiogenesis pathways.[23] miR-1290 has been known to regulate inflammation, cell cycling, and apoptosis, and was suggested to be a prognostic biomarker for GI malignancy.[25] FOXA1, a transcription factor and a potential target gene of miR-1290, has been demonstrated to play important roles in the differentiation of enteroendocrine cells, and maintenance of GI epithelial Muc2 expression and ion channel integrity.[26] Both miR-375 and miR-1246 have been proposed as potential biomarkers for diagnosing inflammatory bowel disease,[27] and the level of circulating miR-1246 could

also reflect the activity of disease in patients with Crohn's disease and patients with ulcerative colitis.[28] Thus, we postulate that these miRNAs could be markedly upregulated in the inflamed bowel of NEC, and were overspilled in large quantities into the circulation.

Our results showed that miR-1290 was upregulated early in the disease process and, thus, fulfilled the criterion as a specific early warning biomarker for NEC. Cases with levels of >650 copies/μL were very likely to be NEC (15 of 18 cases, excluding the 2 hematologic/malignancy-associated cases). Levels between 220 and 650 copies/μL constituted an intermediate zone, and incorporating CRP in the algorithm decreased the number of false-positive NEC cases from 20 to 10, and substantially improved the PPV, LHR+, and DOR. Thirty of the 36 (83%) were correctly identified NEC cases (**Figure 6**). Ten cases were mistakenly classified as NEC, and 6 proven NEC cases escaped detection (details of the misclassified cases were summarized in **Appendix 3** [available at www.jpeds.com]). It is worth noting that the hemophagocytic lymphohistiocytosis and hepatic hemangioendothelioma cases had markedly elevated miR-1290 levels (8741 and 1174 copies/μL), indicating a probable association of miR-1290 with hematologic/malignancy-associated conditions.[29] Because miR-1290 can effectively differentiate both mild medical and severe surgical NEC from neonatal sepsis and enabled 19% of delayed cases to be diagnosed 7.8 to 32.2 hours earlier, its clinical usefulness surpasses conventional circulating protein NEC biomarkers previously described.[23]

There are limitations in the current study. As with any diseases without a gold standard diagnostic tool, there will be some uncertainties on the diagnosis of NEC in a proportion of cases. However, our stringent study design involving objective criteria and consensus of experienced neonatologists and pediatric radiologists minimized the chance of misclassification. Further, all inborn infants were nursed in the neonatal unit until discharged and, thus, cases with bowel strictures or complications secondary to silent NEC would have been detected during hospitalization. Nonetheless, mild gut inflammation without definitive radiologic features of NEC could escape the stringent evaluation. Second, our design standardized the timing of blood collection at the initial sepsis workup; the decision to perform the sepsis screening was subjected to interpretation of clinical signs by the attending neonatologist. In a real-life clinical setting, this represented the quickest and the most ethical timing for obtaining the blood sample before commencement of antibiotics and other treatments. The high sensitivity and specificity, and 12-fold increase in median levels of miR-1290 in NEC vs non-NEC cases confirms the usefulness of miR-1290 for accurately identifying both mild and severe NEC cases, irrespective of the subjective influence of human interpretation of clinical features. Nutritional data were not collected in our original study design because the prime objective of this study was to discover novel and specific early biomarkers for differentiating NEC from sepsis cases, irrespective of their mode and volume of milk feeding. Details of nutritional data in 15 NEC and 23 non-NEC cases are summarized in **Appendix 4** (available at www.jpeds.com). The results do not reveal any correlation between different modes of enteral feeding or volume of milk consumption and miR-1290 levels. In retrospect, because enteral nutrition is an important etiologic factor of NEC,[30] such data might provide insight on the inter-relationship between oral feeding and miRNA regulation or their epigenetic influence on GI tissue.[31] Further, none of the infants received probiotics for prophylaxis against NEC.

In past decades, targeting mediators from known signal pathways has dominated the research approach for biomarker evaluation and use in neonatal sepsis. In this new era of specific biomarker discovery, researchers are equipped with the latest technology to apply a hypothesis-free approach to discover novel biomarkers for surveillance and/or early diagnosis of specific diseases and organ injuries. We have identified miR-1290 to be a specific biomarker for the early diagnosis of both mild medical and severe surgical cases of NEC. A simple 2-stage algorithm using miR-1290 and routine CRP provided the optimal diagnostic characteristics that could differentiate NEC from neonatal sepsis and non-NEC/nonsepsis cases with a high degree of accuracy, and enabled frontline neonatologists to make an earlier diagnosis in both delayed surgical and medical cases. The early response of miR-1290 in the disease process, the requirement of only 0.1 mL of plasma, a short laboratory turnaround time, plus the feasibility of performing the test on an ad hoc basis, render miR-1290 very suitable as an early warning biomarker for NEC for preterm infants. Unlike previous blood protein biomarkers,[23] which could only detect severe surgical cases, miR-1290 is useful in identifying mild medical NEC cases with high precision. miR-1290 and its diagnostic algorithm should be further tested in a large, multicenter study, especially for community-based hospitals, in determining its clinical usefulness for facilitating early transfer of NEC cases. ■

Submitted for publication May 21, 2018; last revision received Aug 31, 2018; accepted Sep 11, 2018

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Appendix 1

Patients and Methods Section: Sample Size of the Cohort Study

The sample size for a 5% error of the sensitivity and specificity values was estimated to require 298 suspected episodes (ie, including NEC, sepsis, clinical sepsis, and non-NEC/nonsepsis cases).

Considering the high mortality and potentially serious morbidity associated with NEC, diagnostic tests with high sensitivity and specificity are most desirable. Thus, we have purposely set both the sensitivity and specificity at 85%. Operationally, an error of 5% is defined as being acceptable for the observed sensitivity and specificity values. Thus, the number of non-NEC cases (ie, groups 2a, 2b, 2c, and 3) required to be studied are:

$$n = \left(\frac{z}{e}\right)^2 pq$$

where n = the number of patients without NEC, z = 99% level of confidence = 2.33, e = error = 0.05, P = level of sensitivity = 0.85, q = 1 - specificity = 0.15.

Number of patients without NEC

$$= \frac{2.33^2 \times 0.85 \times (1-0.85)}{0.05^2} = 277 \text{ cases}$$

Because the disease prevalence was observed to be 7% in our previous studies, the total number of suspected NEC cases required to be studied

$$= \text{number of non-NEC case} \div (1 - \text{prevalence of NEC cases})$$

$$= 277 \div (1 - 0.07)$$

$$= 298 \text{ suspected sepsis/NEC cases}$$

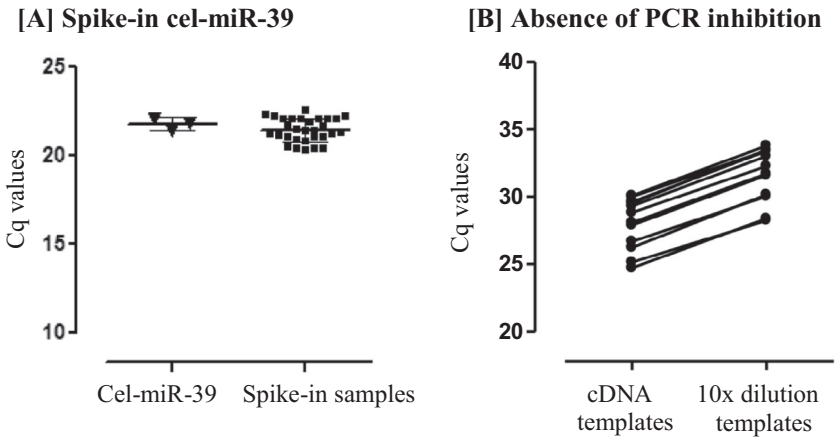
Thus, a minimum of 298 suspected sepsis/NEC cases is required to be recruited into this study.

Appendix 2

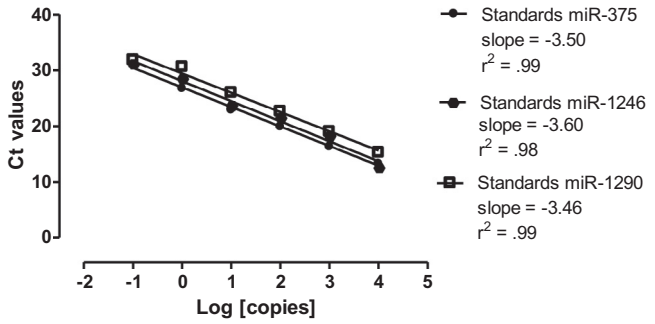
Patients and Methods Section: Plasma Preparation, miRNA Analysis and Validation of Expression by RT-qPCR

Blood samples during the sepsis evaluation (day 0) were collected in prechilled ethylenediaminetetraacetic acid (EDTA) bottles and transported to the laboratory for daily processing. Cell-free plasma was collected by 2-step centrifugation at 1900*g* and 16 000*g*, both for 10 minutes at 4°C. Plasma samples were then stored at -80°C until extraction. Synthetic cel-miR-39 (Integrated DNA Technologies, Singapore, Republic of Singapore) was spiked in to each sample as quality control (**Appendix Figure 1**). The plasma sample (100 µL) was subjected to small RNA purification using the miRNeasy Serum/Plasma Kit (QIAGEN, GmbH, Hilden, Germany).

For microarray analysis, 10 µL of RNA from each specimen was labelled with the FlashTag Biotin RNA Labelling Kit and hybridized to GeneChip miRNA array 4.0, covering 2,578 human mature miRNAs (Affymetrix, Santa Clara, California). Genechips were then washed and stained using the FS450 Fluidic Station (Affymetrix). The fluorescent signal intensity of stained chips was captured by the GeneChip Scanner 3000 7G System (Affymetrix). Data were analyzed using the Partek Genomics Suite v6.5 software (Partek, St. Louis, Missouri). Probeset signals were normalized with the Robust Multi-array Average method with log2-transformation. miRNA



Appendix Figure 1. Quality control of miRNA detection. **A,** Spike-in cel-miR-39 Synthetic cel-miR-39 was spiked into each plasma sample (n = 30) and 3 control tubes were extracted in parallel. Results indicated consistently high extraction efficiency (98.6% ± 3% [mean ± SD], n = 30). **B,** Absence of PCR inhibition was confirmed in miR-1290 miRNA qPCR analyses with 10-fold dilution of 12 samples.



Appendix Figure 2. Standard curves. Standard curves of synthetic miR-375, miR-1246, and miR-1290 showed high efficiency (93%, 90%, 95%) of qPCR amplification.

profiles for NEC, gram-negative septicemia, gram-positive septicemia, and non-NEC/nonsepsis specimens were generated and compared amongst groups (ie, NEC vs gram-negative septicemia, NEC vs gram-positive septicemia, and NEC vs non-NEC/nonsepsis).

For qPCR analysis, cDNA was prepared from 2 μL of RNA from each specimen using the TaqMan MicroRNA Advanced Reverse Transcription Kit and TaqMan Advanced MicroRNA Quantitative (Thermo Fisher Scientific, Austin, Texas). (q)PCR reactions were performed in duplicate with cDNA (5 μL), 2xTaqMan Universal PCR Master Mix (10 μL), and specific primers and probes (final volume 25 μL). Amplification was performed for 40 cycles with denaturation for 15 seconds at 95°C, and annealing extension for 1 minute at 60°C. The emission intensity was detected by the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, California). Parallel runs of synthetic miRNAs (Integrated DNA Technologies, Singapore, Republic of Singapore) at serial dilutions were performed for quantifying the copy number of endogenous miRNAs using the absolute quantification method. Standard curves of these miRNAs are presented in **Appendix Figure 2**. No template controls were found to be undetermined in every run. Intra-assay coefficient of variation of miR-1290 detection was found to be 8.3% at 290 copies/μL and 15.5% at 33 copies/μL (n = 25). The intra-assay coefficient of variation of miR-1246 detection was found to be 6.0% at 599 copies/μL and 15.9% at 38 copies/μL (n = 25). The intra-assay coefficient of variation of miR-375 detection was found to be 4.6% at 274 copies/μL 14.4% at 30 copies/μL (n = 25). These interassay variations should not significantly influent our overall clinical results with a cutoff value of >220 copies/μL.

Appendix 3

Discussion Section: Clinical Characteristics of Misclassified Cases

Ten cases were mistakenly classified as NEC, and 6 proven NEC cases escaped detection. The misclassified cases included hemophagocytic lymphohistiocytosis (n = 1), hepatic hemangioendothelioma (n = 1), previously diagnosed NEC with recurrent distension of bowel loops (n = 2), septic ileus (n = 3), gram-negative septicemia (n = 2), and gram-positive septicemia (n = 1). Both hemophagocytic lymphohistiocytosis and hepatic hemangioendothelioma had markedly elevated miR-1290 levels (8741 and 1174 copies/μL), indicating probable association of miR-1290 with hematologic/malignancy-associated conditions. In cases of previously diagnosed NEC with intermittent dilated bowel loops (343 and 409 copies/μL) and septic ileus (612, 1445, and 2137 copies/μL), elevated miR-1290 levels could have been due to persistent chronic low-grade gut inflammation after partial treatment of NEC or translocation of bacteria in septic ileus via the acutely compromised or even inflamed bowel wall.

Six cases of proven NEC were missed and all had miR1290 of ≤148 copies/mL. Of the 4 medical NEC cases, 1 case had NEC of the stomach (148 copies/mL). Two had very mild manifestations with 1 infant having a nontender abdomen (49 copies/mL) and a normal CRP level (9.7 mg/L), and another with intramural gas resolved within 8 hours of presentation (48 copies/mL). Two surgical NEC cases were not identified. One had pan-necrosis of the bowel but both miR-1290 and CRP levels remained low (73 copies/mL and 4.9 mg/L) and this infant succumbed soon after an open-and-close laparotomy. We speculate that the site (ie, stomach) and severity of bowel tissue damage could influence the plasma level of miR-1290. Also, from time to time, we have encountered that fulminant NEC with pan-necrosis of the bowel could be associated with normal levels of biomarkers. It has been postulated that the source of production of biochemical mediators had been completely destroyed in the pan-necrotic process.[1]

References

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Appendix 4

Discussion Section: Nutritional data of NEC and Non-NEC Cases

Detailed nutrition data of 15 NEC and 23 non-NEC cases were collected. The modes of milk feeding, including nil per os:breast:mixed breast/formula:formula were 0:6:3:6 vs 2:11:5:5 for the NEC vs non-NEC groups, respectively. Volumes of enteral feeding median (range) were 130 mL/kg/d (14-165 mL/kg/d) vs 101 mL/kg/d (0-161 mL/kg/d) for the NEC vs non-NEC groups. Plasma miR-1290 (<100 mL/kg/d) were 684 copies/μL (223-1146 copies/μL) vs 46 copies/μL (6-61 copies/μL), and (≥100 mL/kg/d) were 872 copies/μL (221-5216 copies/μL) vs 37 copies/μL (9-112 copies/μL) for the NEC vs non-NEC groups, respectively. As expected and in accordance with general observations that NEC usually occurred when infants were approaching full enteral feeding, whereas sepsis could happen at any stage of their hospital

stay, it was not surprising that the volume of milk feeding in the NEC group was higher. More important, our data indicated that plasma miR-1290 remained very low even in infants having ≥100 mL/kg/d milk feedings (ie, irrespective of enteral feeding) in the non-NEC group. The available data suggested that the usefulness of miR-1290 as an early biomarker was unlikely to depend on enteral milk intake of preterm infants.

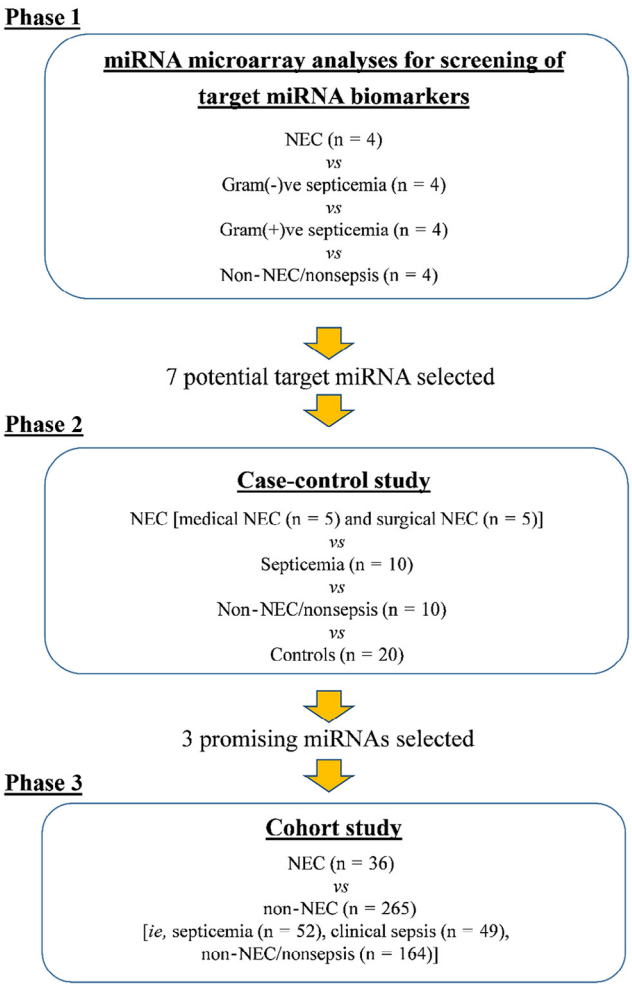


Figure 1. Flow chart of the study plan.

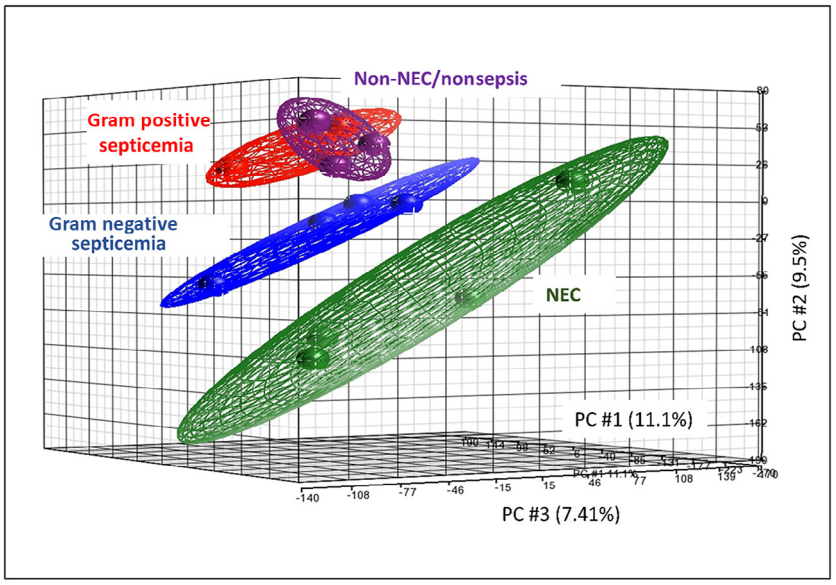


Figure 2. Principal component analysis of miRNA array. Principal component analysis of miRNA expression data revealed distinct clustering of 4 NEC cases (n = 4) separated from gram-negative septicemic cases (n = 4), gram-positive septicemic cases (n = 4), and non-NEC/nonsepsis cases (BPD, n = 3; patent ductus arteriosus, n = 1). Each dot represents a sample and colored according to the respective groups. The percentage of variance for the three principal components were indicated in the axis (principal component PC#1, 2, and 3).

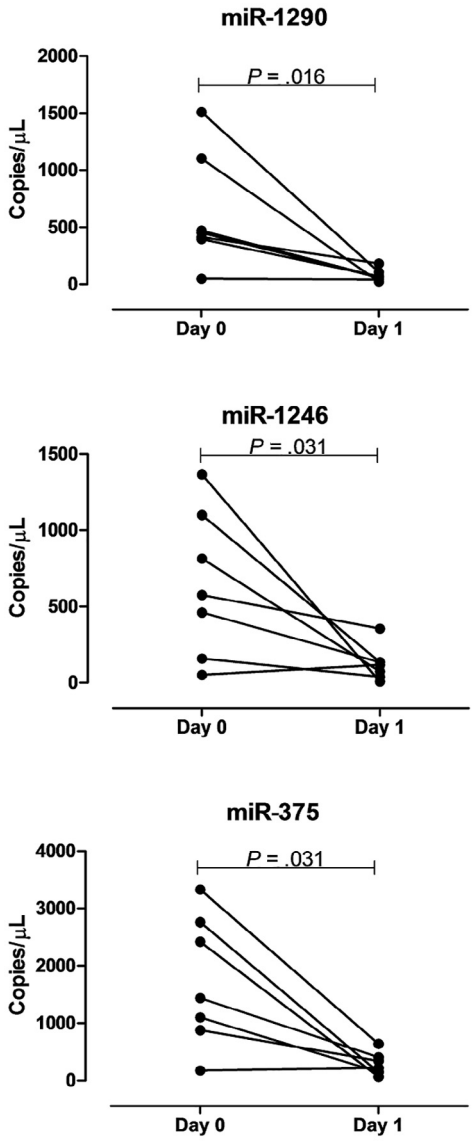
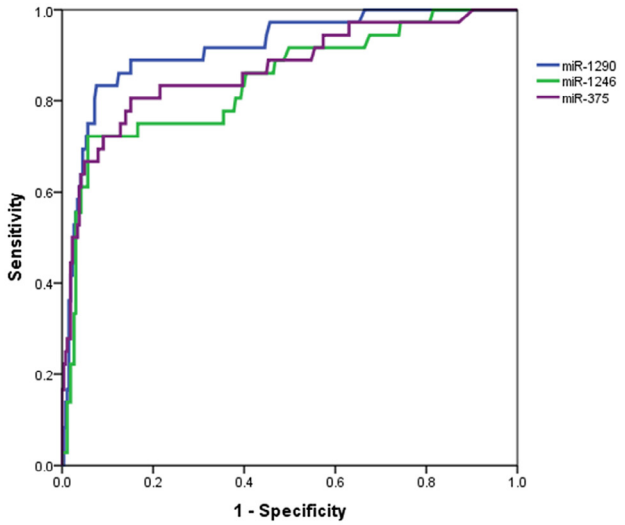


Figure 4. Plasma miRNA expression on days 0 and 1 in infants with NEC. Circulating levels of miR-1290, miR-1246, and miR-375 were significantly decreased in day 1 samples of infants with NEC compared with corresponding samples on day 0 (n = 7).



Targets	AUC	*P* value	95% CI	
			Lower Bound	Upper Bound
miR-1290	0.917	<.001	0.866	0.968
miR-1246	0.843	<.001	0.766	0.921
miR-375	0.869	<.001	0.796	0.942

Figure 5. ROC curves of target miRNAs. ROC curves of miR-1290, miR-1246, and miR-375 were data analyzed from the cohort study comprising 265 non-NEC (ie, gram-negative sepsis, n = 19; gram-positive sepsis, n = 33; clinical sepsis, n = 49; and non-NEC/nonsepsis, n = 164; total N = 265 cases) and 36 NEC cases. The area under the curve, 95% CI, and *P* values were listed for each miRNA. miR-1290 has the largest area under the curve (0.917) compared with miR-1246 (0.843) and miR-375 (0.869).

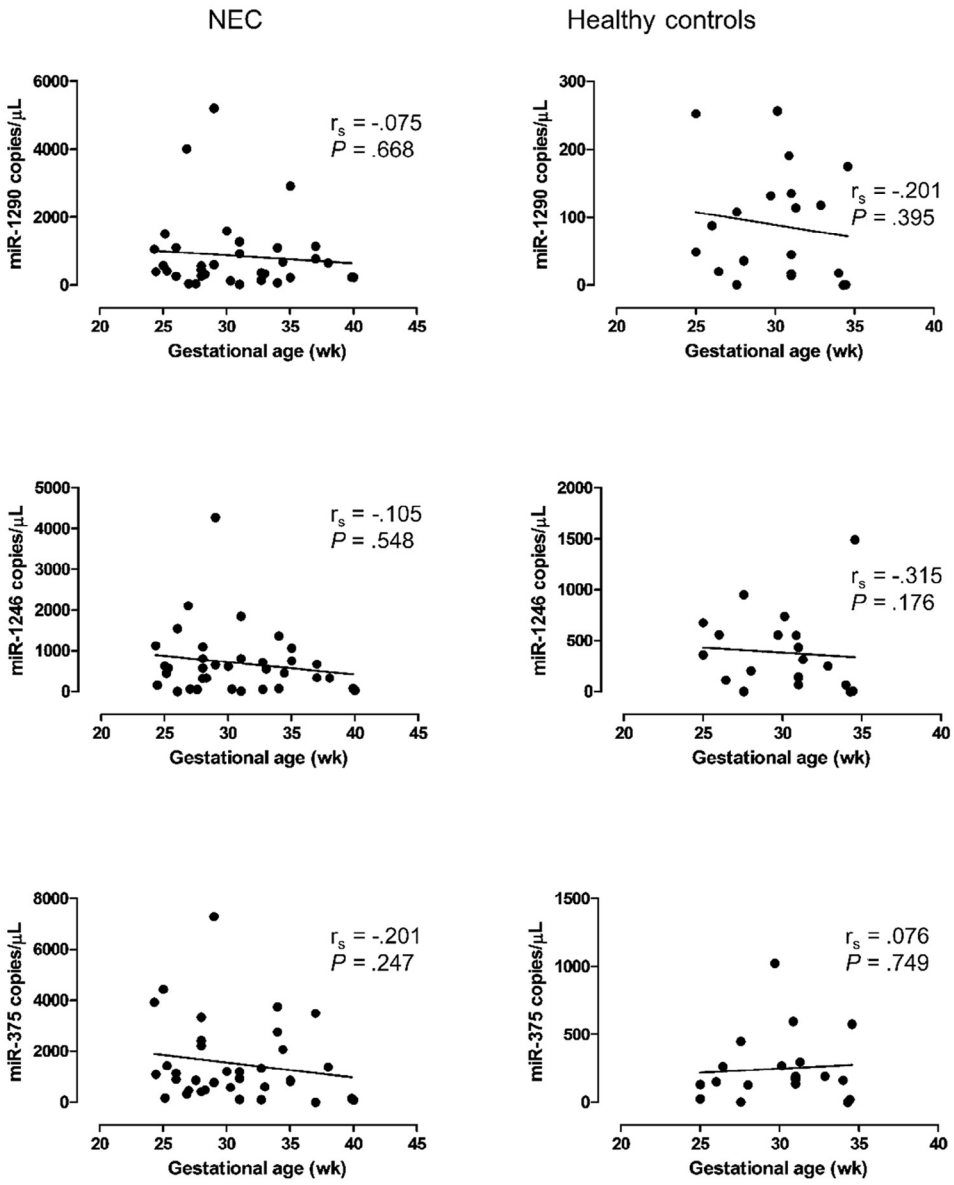


Figure 7. Correlation of miRNA levels and gestational age in NEC and healthy control infants. Spearman correlation coefficient was used to assess relationships between plasma miRNA levels and gestational age of NEC (n = 35) or healthy control groups (n = 20).

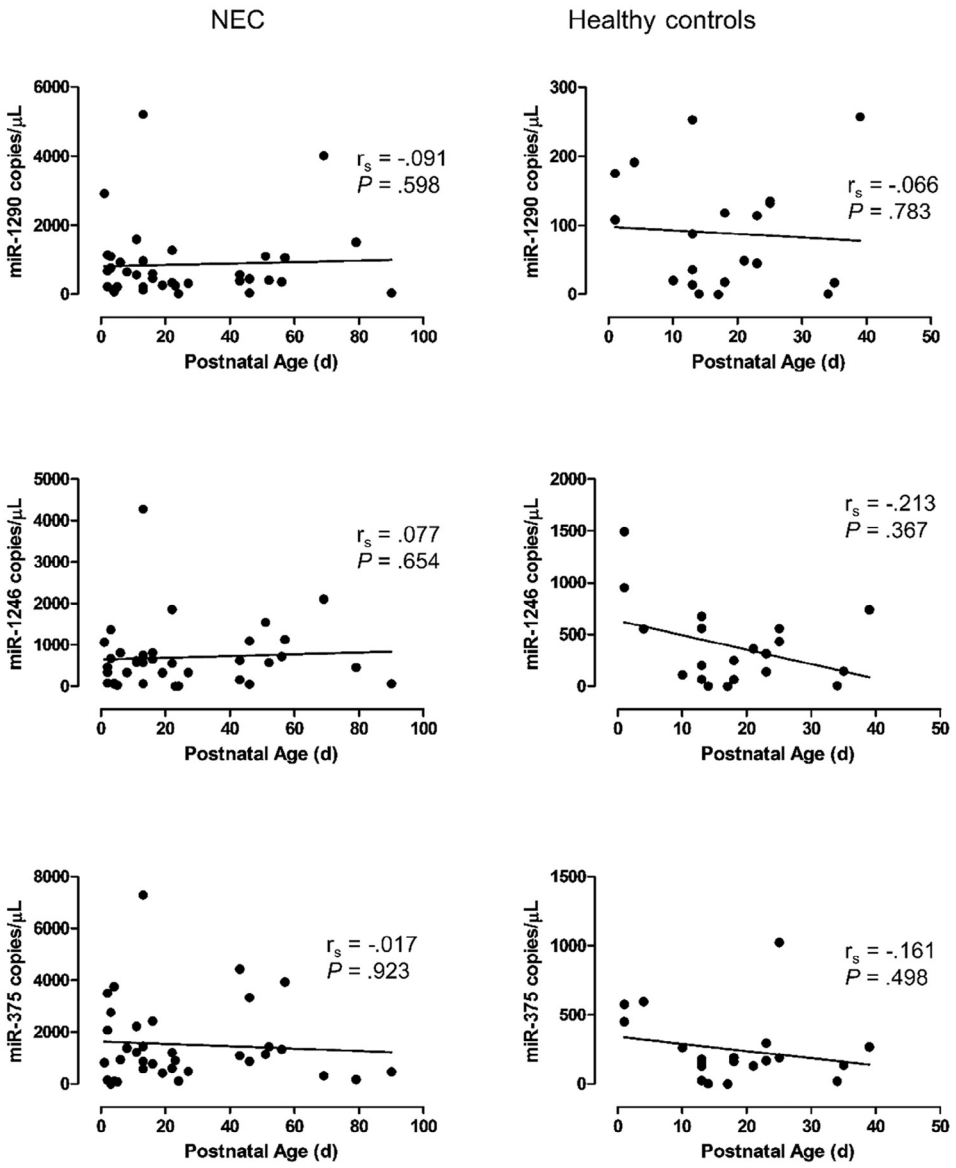


Figure 8. Correlation of miRNA levels and postnatal age at onset of illness in NEC and healthy control infants. Spearman correlation coefficient was used to assess relationships between plasma miRNA levels and postnatal age of NEC (n = 36) at the time of sepsis workup and healthy "controls" infants (n = 20).

Table I. Selection of miRNA targets derived from microarray analysis

Transcript IDs	NEC vs gram-negative sepsis (fold change)	*P* values	NEC vs gram-positive sepsis (fold change)	*P* values	NEC vs non-NEC/nonsepsis (fold change)	*P* values	NEC vs gram-negative, gram-positive and non-NEC/nonsepsis (fold change)	*P* values
hsa-mir-1290	36.753	.000	14.262	.002	34.883	.000	26.345	.000
hsa-mir-1246	46.126	.001	15.919	.006	24.066	.003	26.047	.001
hsa-mir-1233-5p	6.509	.001	9.630	.000	7.865	.001	7.900	.000
hsa-mir-619-5p	2.145	.304	9.126	.013	20.340	.002	7.357	.008
hsa-mir-1469	3.572	.038	13.055	.001	7.993	.004	7.197	.002
hsa-mir-375	6.305	.016	6.488	.017	6.984	.014	6.586	.006
hsa-mir-665	3.811	.037	6.870	.007	5.726	.013	5.313	.006

Table II. Clinical characteristics of infants in the case-control study

		Non-NEC groups			*P* values		
	NEC	Septicemia	Non-NEC/ nonsepsis	Controls	NEC vs septicemia	NEC vs non-NEC/nonsepsis	NEC vs controls
Episodes, n	10	10	10	20	—	—	—
Sex, male	6 (60)	5 (50)	4 (40)	7 (35)	.656	.371	.255
Gestational age, wk	28 (28-29)	31 (29-33)	30 (27-30)	31 (28-32)	.023*	.436	.214
Birthweight, g	1365 (1000-1533)	1475 (1298-1863)	1195 (986-1547)	1377 (905-1646)	.315	.684	.779
Apgar scores							
1 min	8 (7-9)	8 (6-8)	7 (5-8)	8 (7-9)	.796	.143	.983
5 min	9 (8-9)	9 (8-9)	7 (6-9)	9 (8-10)	.912	.043*	1.000
Postnatal age at onset of illness, d	16 (13-23)	25 (17-52)	22 (15-31)	18 (13-25)	.165	.315	.622
Duration of hospitalization (inborns), d	100 (80-199)	69 (54-78)	65 (55-110)	75 (48-90)	.016	.055	.047*
Disseminated intravascular coagulation	5 (50)	4 (40)	0 (0)	0 (0)	1.000	.033*	.002
Died	1 (10)	0 (0)	0 (0)	0 (0)	1.000	1.000	.333

Results are expressed as n (%) or median (IQR).
*Not statistically significant after Bonferroni correction.

Table III. Clinical characteristics of the NEC and non-NEC groups

				Non-NEC groups				*P* values			
	NEC (group 1)	Combined non-NEC (groups 2a, b, c and 3)	NEC vs combined non-NEC (*P* values)	Gram-negative sepsis (group 2a)	Gram-positive sepsis (group 2b)	Clinical sepsis (group 2c)	Non-NEC/ nonsepsis (group 3)	NEC vs gram-negative sepsis	NEC vs gram-positive sepsis	NEC vs clinical sepsis	NEC vs non-NEC/ nonsepsis
Infants, n	35*	197	—	19	32	38	134	—	—	—	—
No. of outborn infants	17	24	—	2	4	7	14	—	—	—	—
No. of episodes	36	265	—	19	33	49	164	—	—	—	—
Sex, male	20 (57)	109 (55)	.842	10 (53)	18 (56)	24 (63)	75 (56)	.750	.941	.600	.901
Gestational age, wk	30 (27-34)	30 (28-33)	.862	30 (28-33)	32 (29-34)	31 (27-35)	29 (27-32)	.568	.230	.449	.549
Birthweight, g	1430 (908-2150)	1410 (988-1790)	.609	1410 (1170-1720)	1625 (1113-1870)	1580 (1045-2008)	1285 (907-1714)	.957	.506	.631	.201
Apgar scores			.469							.277	.298
1 min	7 (6-8)	7 (5-8)		8 (7-9)	8 (6-9)	7 (5-8)	7 (5-8)	.244	.749		
5 min	9 (8-9)	8 (7-9)	.563	9 (8-10)	9 (8-10)	8 (7-9)	8 (7-9)	.328	.396	.254	.355
Postnatal age at onset of illness, d	16 (5-45)	27 (17-51)	.005	24 (9-43)	28 (19-56)	30 (21-61)	25 (15-47)	.589	.005	.002	.015‡
Duration of hospitalization (inborns), d	93 (79-151)	79 (53-112)	.096	75 (50-132)	80 (55-126)	97 (61-143)	82 (57-123)	.303	.171	.959	.221
DIC, n	17 (47)	36 (14)	<.001	17 (89)	7 (21)	6 (12)	6 (4)	.002	.023‡	<.001	<.001
Died, n	4† (11)	3 (2)	.011	0 (0)	0 (0)	1 (3)	2 (1)	.285	.115	.187	.017‡

*One infant had recurrent NEC.
†Three infants died of NEC and 1 infant died of subsequent infection at the age of 8 months.
‡Not statistically significant after Bonferroni correction.
Results are expressed as n (%) or median (IQR).