Diagnostic and prognostic values of miR181b-5p and miR21-5p for neonatal sepsis risk and their link to SNAP II score and disease mortality

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\textbf{ABSTRACT}

\textit{Background:} Neonatal sepsis is a lethal syndrome that necessitates prompt treatment to avoid disease complications. As a result, biomarkers that may either differentiate sepsis early or predict the outcome of sepsis are essential.

\textit{Aim:} The goal of this research was to find out the clinical weight of using miR181b-5p and miR21-5p expression levels as diagnostic and prognostic new genetic markers for neonatal sepsis.

\textit{Method:} A total of 60 neonates with sepsis and 60 healthy neonates were involved in this study. Laboratory tests include complete blood count (CBC), random blood sugar (RBS), arterial blood gases (ABG), and serum C-reactive protein (CRP). Neonates with sepsis were assessed by the Score for Neonatal Acute Physiology II (SNAP II). The serum fold changes of the target miRNAs were determined using qRT-PCR and the \(2^{-\Delta\Delta Ct}\) equation.

\textit{Results:} The relative serum level of miR181b-5p was [median (IQR) = 0.2509 (0.0009–4.11)] and for miR21-5p was [median (IQR) = 0.07 (0.007–7.16)] which were significantly downregulated in patients with neonatal sepsis compared to controls (\(p < 0.001\) each). There was a strong significant positive correlation between miR181b-5p and miR21-5p with \(r = 0.718\) and \(p < 0.001\). MiR181b-5p and miR21-5p were significantly negatively correlated with total leucocytic count (TLC), lymphocytic count, and CRP. While they were both positively correlated to the SNAP II score. Obvious association between higher expressions of target genes and higher SNAP II score groups. After a following-up period, twenty-two (36.7%) neonates died, while 38 (63.3%) of the babies became better and were released from the hospital. We reported that miR-181-5p, miR-21-5p, SNAP II score and CRP were significantly higher in non-survivors than survivors. Only miR181b-5p, miR21-5p, and SNAP II were predictive factors of septic mortality.

\textit{Conclusion:} MiR181b-5p and miR21-5p are diagnostic and prognostic biomarkers of neonatal sepsis.

1. Introduction

Neonatal sepsis (NS) is a lethal syndrome that may lead to multiple organ failures. NS results from a blood infection in neonates aged 28 days or younger \cite{1}. Even though NS is a preventable disease, it is a global health burden, particularly in developing nations, where it accounts for around one million neonatal deaths each year, with survivors facing long-term disability \cite{2}. According to a recent study conducted in Egypt, 21.5% of preterm newborns hospitalized in neonatal intensive care units (NICU) during the study period had NS, with a mortality rate of 51.6% \cite{3}.

Unfavorable outcomes of NS in developing countries may be due to a lack of protective procedures such as health care facilities, expert health staff, and sufficient infection control protocols \cite{1}, so early detection...
and treatment of NS is the second line of defense to enhance the therapeutic choices and decrease disease consequences in these countries.

NS clinical picture is elusive and vague affecting more than one system or organ resulting in misleading multisystem signs and symptoms, that can interfere with other life-threatening conditions, such as respiratory distress syndrome, necrotizing enterocolitis, and intracranial hemorrhage. Also, blood culture, the principal test for definite diagnosis of NS, needs approximately 24–48 h to deliver the results with possible false-negative and false-positive results. In addition, currently used biological markers for instance, C-reactive protein (CRP), interleukin-6 (IL-6), procalcitonin (PCT), and erythrocyte sedimentation rate (ESR) are highly sensitive but could not differentiate between infectious and non-infectious inflammatory responses [19].

As the limitation of the currently established diagnostic measures, the search for likely diagnostics of new circulating accessible genetic markers expands, also, the probability of linking these gene expressions with susceptibility and clinicopathological features of NS for using them as diagnostic/prognostic parameters is being investigated including microRNAs.

MicroRNAs (miRNAs) are short non-coding RNA transcripts that have crucial roles in regulating host immune response and underlying inflammation by influencing the expression of related genes [4]. Significantly, miRNAs can be released in the extracellular compartments where they can be easily detected in serum [5].

To date, numerous previous studies extensively examined the association and molecular functions of miR181b and miR21 in the pathogenesis, the prognosis of vascular inflammation and sepsis both in vitro and in vivo [6,7]; miR181b-5p and miR21-5p have been shown to play a critical role in sepsis pathology as regulatory molecules for the NF-κB pathway, which is an essential inflammatory pathway [8]. Also, both genes affect the regulation of inflammatory cytokines, chemokines, and adhesion molecules [9,10]. Besides, they play a crucial role in immune response regulation [11]. Moreover, they are diagnostic and prognostic markers in adult septic models [6,10] and animal models [12], but, there is a lack of research discussing their value in NS. In this study, we compare serum miRNAs levels (181b-5p and 21-5p) of NS patients to healthy neonates as well as their linkage to SNAP II mortality score aiming at exploring the clinical weight of using miR181b-5p and miR21-5p expression levels as diagnostic and prognostic new genetic markers for NS.

2. Subjects & methods

2.1. Subjects & type of the study

This prospective case-control study was executed per Helsinki’s ethics for medical research [13] and its procedures were approved by the Ethics Committee of El- Fayoum University (El-Fayoum, Egypt) protocol no. (R211-session 89). Written informed consent was gotten from the parents of the neonates. The current study involved 120 neonates recruited during the period from Jan 2022 to June 2022 and they were two groups: first, the septic group with sixty newborns who were intensive care unit patients in the Pediatrics Department at El-Fayoum University Hospital. Second, the control group includes sixty healthy neonates with no clinical picture suggestive of sepsis.

2.2. Inclusion and exclusion criteria

Inclusion criteria include neonates with age ≤28 days, birth weight of >1000 gm, and a gestational age ≥28 weeks who suffered from early-onset (1–3 days) or late-onset (>3 days) signs and/or symptoms of sepsis. If a neonate in the study exhibited any of the following manifestations; poor reflexes, lethargy, abdominal distension, diarrhea, blood in stool, respiratory distress; apnea, grunting, cyanosis, brady or tachycardia, seizures, bulging fontanel, reduced sucking, dippled eye, hyper or hypothermia ≥37.5C or <35.5C respectively; he or she was suspected of having sepsis which is confirmed by blood culture.

Exclusion criteria include neonates with congenital anomalies including, renal, cardiac, hepatic, or CNS anomalies, chromosomal anomalies, intramural growth retardation, neonates whose mothers suffered from autoimmune diseases, diabetes, or hypertension, and neonates having severe perinatal asphyxia to enroll that respiratory distress that develops later is on top of sepsis, neonates died within one day after inclusion were excluded from the study, the justification for excluding neonates who died within the first day of enrolment was that these babies would be seriously ill at the time of admission, and any further usefulness provided by an illness severity score in such babies seemed doubtful.

2.3. Full history, clinical examination, and laboratory investigations

Full history from involved subjects’ sheets are documented including: age, sex, birth weight, gestational age, mode of delivery, premature rupture of membranes (PROM), and maternal risk factors of NS (history of prolonged labor, febrile illness of the mother two weeks before delivery, meconium-stained amniotic fluid and foul-smelling discharge of amniotic fluid, inadequate pre and post-partum hygiene or maternal invasive medical procedure). Out of sixty patients, 40 (66.67%) were premature <37 weeks, 39 (65.00%) with low birth weight <2.5 kg and 18 (30%) with PROM, and 3 (5%) with maternal pyrexia two weeks before delivery. There are no other risk factors detected in our patients.

Full clinical examination for all participants was done including newborn measurements (head circumference, weight, length), normal primitive neonatal reflexes assessment, vital signs, and Apgar score at 1 min. Diagnosis of sepsis depended on the criteria demarcated at the 2003 Kunming Neonatal Sepsis Definitions Conference [14], which accounts for clinical manifestations of sepsis in preterm or term babies together with positive blood culture.

Laboratory tests were done including complete blood count (CBC), random blood sugar (RBS), and arterial blood gases (ABG). The Score for Neonatal Acute Physiology II (SNAP II) was done for all newborns with sepsis and is considered an indicator of mortality [15]. SNAP II is a physiologically-built score that is dependent on six items that are measured within the initial 12 h of admission (mean blood pressure ‘mm Hg’, lowest temperature, Po2/Fio2 ratio, serum pH, urine output “ml/kg/hr” and multiple seizures) [16]. The severity of the disease was graded according to this score (mild: 1–20, moderate: 21–30, severe: 31–40, critical: >40).

2.4. Outcomes of NS

Neonates with sepsis were further followed up for 20 days to report whether improved and discharged or died. Twenty-two (36.7%) neonates died within this period due to septicemia, while 38 (63.3%) babies improved and left the hospital. The majority of non-survivor patients 12 (54.8%) died from respiratory failure, 4 (18%) died from multiple organ dysfunction syndromes (MODS) and had peritoneal dialysis, 3 (13.6%) died from disseminated intravascular coagulopathy (DIC), and 3 (13.6%) died from hepatic failure. CPAP and traditional mechanical ventilation were used in the trial of resuscitation of cases, however, ECMO was not available.

3. Methods

3.1. Sample preparation

We collected a total of 5.0 ml of venous blood from the neonates with sepsis when they were diagnosed, 3.0 ml was collected into a plain tube and was kept at room temperature for 15 min to allow the serum to clot. Then the upper supernatants were collected and centrifuged at 4000 xg for 10 min to separate serum, part of the separated sera (1ml) was used...
for detection of CRP. And the remaining parts were stored at -80 °C for later uses in RNA extraction. One ml was collected in an EDTA tube for CBC. The remaining 1.0 ml of whole blood samples were drawn for automated blood culture using BC bottles (Peds Plus; Becton Dickinson). For the control group, 4.0 ml of venous blood samples from the control group were obtained during routine consultation for scheduled vaccination, and each sample was divided as follows; 3.0 ml for serum separation (miRNAs extraction and CRP measurement) and 1.0 ml for CBC.

3.2. Blood culture processing

The BACTEC blood culture system 9050 was used (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA). From each positive blood culture bottle, a subculture was done according to the laboratory operating procedure. Antimicrobial sensitivity testing was done by the Kirby–Bauer disc diffusion technique and the interpretation of the result was per Clinical Laboratory Standards Institute (CLSI) guidelines [17]. To ensure the quality and accuracy of the testing processes, Staph aureus ATCC 25923 and Escherichia coli ATCC 25922 were utilized as controls. The results of the blood culture are in (Table 1).

3.3. Total RNA extraction including miRNAs

Extraction of total RNA (including miRNAs) from the serum was done using the miRNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s procedure. RNA extraction steps included; we mixed 1000 μL of QIAzol lysis reagent with 200 μL of sample and incubated the mixture for 5 min at room temperature. After that, we added 200 μL of chloroform to the mixture, shook the tube forcefully, and centrifuged it at 12,000xg for 15 min at 4 °C. Chloroform separated the homogenate into three layers: an upper aqueous layer containing RNA, an intermediate layer containing DNA, and a third lower organic layer containing denatured proteins. To create proper binding conditions for all RNA molecules, we removed the aqueous layer on top and added 1.5 times its volume of 100% ethanol. The sample was then passed down the RNeasy Mini spin column, we utilized the kit’s washing buffers (RWT and RPF), in which total RNA bound to the washing buffers (RWT and RPF), in which total RNA bound to the sample size formula:

\[
\text{Necessary sample size} = \left( \frac{Z - \text{Score}}{\text{SD} \times (1 - \text{SD})} \right) \times \text{margin of error}^2
\]

Confidence levels of 95% are converted into Z scores, the standard deviation is 0.5, and the margin of error is 0.05. The sample size equals 60 cases.

3.6. Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 24 was used to analyze data. Quantitative data were represented by the minimum and maximum, mean, median, standard deviation (SD), and standard error of the mean (SEM). Categorical data were shown as frequency and percentage. Nonparametric data (categorical data) were compared by the Chi-squared test. The Spearman correlation coefficient was used for correlations among four markers and between markers and quantitative variables. The multivariate stepwise logistic regression was constructed to identify the significant predictors of cerebral stroke among the four markers. The receiver operating characteristic (ROC) curve was performed with the area under curve (AUC) analysis to detect the best cutoff value of the four tested markers for stroke detection. For all tests, P-value < 0.05 was considered statistically significant.
4. Results

4.1. Demographic, clinical, and laboratory data of neonates with sepsis and controls

A total of 60 neonates with sepsis and 60 healthy neonates were involved in this study. The history, risk factors, and clinical characteristics, including age, sex, gestational age, mode of delivery, neonatal measurements, vital signs, Apgar score at 1 min, and laboratory data involving CBC and ABG were summarized in Table 2. Patients with NS were characterized by significantly lower gestational age and hence a significantly higher percentage of prematurity, and a significantly higher percentage of PROM. Regarding clinical parameters, vital signs (higher temperature, respiratory rate, and heart rate but lower mean arterial blood pressure), lower Apgar score at 1 min, CBC (higher TLC, red cell distribution width (RDW), lymphocytic count, and random blood sugar (RBS), but lower platelet count and hemoglobin concentration), and more acidic blood, and higher CRP were detected in patients with sepsis than controls.

4.2. Serum miR181b-5p and miR21-5p status in patients with NS

As presented in (Table 3, Fig. 1), the relative serum level of miR181b-5p was [median (IQR) = 0.2509 (0.0009–4.11)] and for miR21-5p was [median (IQR) = 0.07 (0.007–7.16)] which were significantly down-regulated in patients with NS compared with those in the controls (P < 0.001 each).

4.3. Pearson correlation between miR181b-5p and miR21-5p with the clinical variables of NS

There was a strong significant positive correlation between miR181b-5p and miR21-5p with r = 0.273, p = 0.035 for miR181b-5p and r = −0.339, p = 0.008 for miR21-5p, lymphocytic count (r = −0.394, p = 0.002 for miR181b-5p and r = −0.259, p = 0.046 for miR21-5p), and CRP (r = −0.300, p = 0.020 for miR181b-5p and r = −0.280, p = 0.030 for miR21-5p). While, they were positively correlated to SNAP II score (r = 0.305, p = 0.018 for miR181b-5p and r = 0.276, p = 0.033 for miR21-5p). MiR181b-5p was borderline positively correlated with RBS (p = 0.047) (Table 4, Fig. 2).

4.4. The association between both miR181b-5p and miR21-5p, risk factors, and the clinical presentation of NS

Regarding risk factors, lower target genes (miR181b-5p and miR21-5p) were significantly associated with prematurity (p = 0.04 & 0.03 respectively). Also, lower miR21-5p was just signed with low birth weight (p = 0.05). The most clinical presentation in neonatal septic patients was poor reflexes (93.33%), followed by respiratory distress (81.66), then fever (76.67), bleeding (25%), convulsions, and abdominal distension (18.33% each), apnea (13.33%), and finally lethargy and bradycardia (6.67%). There was no significant association between clinical signs of NS and the fold changes of target genes (miR181b-5p and miR21-5p) (Table 5).

4.5. Association analysis between the fold change of miR181b-5p and miR21-5p with the age of onset of sepsis and SNAP II score in the NS group

Two groups of sepsis-affected neonates were identified, neonates with early-onset sepsis (EOS) (within 1–3 days) which included 26 neonates (43.3%), and those with late-onset sepsis (LOS) ((>3 days) which included 34 neonates (56.7%). On testing the relationship between these groups and target genes; no statistical significance was detected between the two groups regarding the fold change of miR181b-5p and miR21-5p (Table 6).

The study NS population had a median SNAP II score of 9 (IQR = 0–22.5). Thirty-four (56.7%) neonates had mild illness [SNAP II < 30], 10 (16.7%) had moderate illness [SNAP II ≥ 30–60], and 8 (13%) had severe illness [SNAP II > 60]. The association between the fold change of miR181b-5p and miR21-5p with the age of onset of sepsis and SNAP II score in the NS group is presented in Table 6.

Table 2

| variables | Control (N = 60) | Neonatal sepsis (N = 60) | P-value |
|-----------|---------------|-------------------------|---------|
| Age and gender | Age/days | 8.6 ± 6.69 (1–30) | 8.92 ± 8.65 (1–29) | 0.832 |
| | Male n (%) | 21 (35.0%) | 23 (38.33%) | 0.744 |
| | Female n (%) | 39 (65.0%) | 37 (61.67%) | <0.0001 |
| Demographic age/weeks | Gestational age/weeks | 37.65 ± 0.57 | 36.35 ± 1.71 | <0.0001 |
| Mode of delivery | NVD n (%) | 10 (16.67%) | 15 (25.0%) | 0.651 |
| | CS n (%) | 50 (83.33%) | 45 (75.0%) | 0.482 |
| Risk Factors | PROM | 0.0 (0.0%) | 18 (30.0%) | <0.0001 |
| | Prematurity <37GW n (%) | 18 (30.0) | 40 (66.67%) | <0.0001 |
| | Low birth weight <2.5 kg; n (%) | 28 (46.67) | 39 (65.0%) | 0.059 |
| | Maternal pyrexia 2 weeks before delivery n (%) | 1 (1.67) | 3 (5.0%) | 0.339 |
| Measurements | Birth weight/kg | 2.66 ± 0.41 | 2.48 ± 0.47 | 0.023 |
| | Length (cm) | 48.03 ± 1.75 | 47.3 ± 3.21 | 0.124 |
| | HC (cm) | 33.17 ± 1.01 | 32.86 ± 1.33 | 0.186 |
| | Temperature | 36.99 ± 0.07 | 37.82 ± 0.73 | <0.0001 |
| | RR/ min | 43.32 ± 3.28 | 64.17 ± 12.82 | <0.0001 |
| | HR/ min | 115.42 ± 11.69 | 137.02 ± 15.53 | <0.0001 |
| | MAP mm Hg | 63.44 ± 9.07 | 51.65 ± 9.95 | <0.0001 |
| | Apgar Score at 1 Min | 9.32 ± 0.67 | 8.72 ± 1.06 | <0.0001 |
| | CBC | TLC/ mm³³ | 1047.3 ± 2994.5 | 1745.67 ± 8407.6 | <0.0001 |
| | Platelets (<10⁹/ L) | 335466 ± 58277.9 | 238133.3 ± 181047.2 | <0.0001 |
| | RDW % | 4.16 ± 0.57 | 15.70 ± 1.27 | <0.0001 |
| | Lymph (10³/µL) | 3666.67 ± 1091.5 | 7183.3 ± 4981.6 | <0.0001 |
| | Hb (mg/dL) | 13.67 ± 2.08 | 11.13 ± 2.16 | <0.0001 |
| | CRP (mg/dl) | 0.324 ± 0.04 | 41.1 ± 29.25 | <0.0001 |
| | PH | 7.35 ± 0.10 | 7.23 ± 0.15 | <0.0001 |
| | CO2 | 41.83 ± 5.97 | 40.37 ± 12.64 | 0.418 |
| | HCO3 | 19.81 ± 2.09 | 17.76 ± 1.61 | <0.0001 |

Data are expressed as mean ± SD or n (%). PROM: premature rupture of membrane, NVD: normal vaginal delivery, CS: cesarean section, HC: head circumference, BW: birth weight, RR: respiratory rate, HR: heart rate, MAP: mean arterial pressure, TLC: total leucocytic count, RDW: red cell distribution width, HB: hemoglobin, RBS: random blood sugar, CBC: complete blood count, CRP: C reactive protein.

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critical illness [SNAP II > 40], On testing the relationship between these groups and target genes; statistically significant differences were detected between groups regarding fold change of miR181b-5p and miR21-5p with an obvious association between higher expressions of target genes and higher SNAP II score group (Table 6).

### 4.6. Differences between survivors and non-survivors regarding miR181b-5p and miR21-5p expression levels, SNAP II score, and CRP level

After a following-up period reached twenty days, twenty-two (36.7%) neonates died within this period due to septicemia, while 38 (63.3%) babies improved and were released from the hospital and on examination of miRNA expression levels, and SNAP II and CRP between survivors and non-survivors; we reported that miR-181-5p, miR21-5p, SNAP II score and CRP were significantly higher in non-survivors than survivors (Table 7, Fig. 3).

### 4.7. Univariate and multivariate logistic regression analyses

Logistic regression analysis to obtain independent risk factors for sepsis mortality was done in two steps; first univariate analysis detected that miR181b-5p, miR21-5p, CRP, TLC, and SNAP II were significantly different between sepsis survivors and non-survivors (p < 0.05) (Table 8).

Second, we performed multivariable logistic regression analysis using variables that showed significance in univariate logistic regression analysis, only miR181b-5p, miR21-5p, and SNAP II were predictive factors of septic mortality (Table 9).
4.8. ROC curve analysis to detect the diagnostic and prognostic performance of target genes altogether with SNAP II

We performed ROC curve analysis to elucidate the diagnostic values of the miRNAs, and the area under the curve (AUC) was 0.917 (0.847–0.987) for miR21-5p and 0.833 (0.739–0.928) for miR181b-5p with cutoff = 0.067 and 0.324 respectively with total accuracy reaches nearly 96% for each. Also, ROC curve analysis was created to reveal the

Table 5
The association between both of miR181b-5p and miR21-5p, risk factors & the clinical signs of neonatal sepsis using Chi-squared test and Exact-Fisher test.

| Clinical signs                        | n (%) |           |           |           |           |
|---------------------------------------|-------|-----------|-----------|-----------|-----------|
|                                       |       | miR181b-5p|           | miR21-5p  |           |
|                                       |       | Median (IQR) | P | Median (IQR) | P |
| PROM                                  | Yes   | 18 (30.0)  | 0.29 (0.003–2.87)  | 0.307 | 0.17 (0.006–1.99)  | 0.269 |
|                                       | No    | 42 (70.0)  | 0.01 (0.009–2.09)  | 0.36 (0.007–2.04) | 0.03 |
| Gestational age/weeks                 | Premature <37W | 40 (66.67) | 0.05 (0.009–0.98) | 0.36 | 0.01 (0.007–0.99) | 0.03 |
|                                       | Full term | 20 (33.33) | 0.18 (0.08–1.87)  | 0.33 (0.03–1.08) | 0.05 |
| Birth weight/kg                       | Low <2.5 kg | 39 (65.0)  | 0.03 (0.07–0.96)  | 0.01 (0.09–0.75) | 0.05 |
|                                       | Average | 21 (35.00) | 0.15 (0.07–0.87)  | 0.19 (0.05–0.86) | 0.225 |
| Febrile illness of mother 2W before delivery | Yes | 3 (5.0)  | 0.01 (0.03–1.54)  | 0.178 | 0.15 (0.04–0.77) | 0.225 |
|                                       | No    | 57 (95.0)  | 0.17 (0.07–1.09)  | 0.01 (0.08–0.97) | 0.230 |
| Reflexes                              | Fair   | 4 (6.67)   | 0.09 (0.0009–0.72) | 0.191 | 0.07 (0.007–0.58) | 0.722 |
|                                       | Poor   | 56 (93.33) | 0.13 (0.07–1.054) | 0.21 (0.03–1.13) | 0.052 |
| Lethargy                              | Yes    | 4 (6.67)   | 0.15 (0.09–0.48)  | 0.337 | 0.02 (0.009–0.85) | 0.099 |
|                                       | No     | 56 (93.33) | 0.04 (0.009–0.24) | 0.11 (0.01–0.92) | 0.05 |
| Convulsion                            | Yes    | 11 (18.33) | 0.03 (0.002–0.37) | 0.158 | 0.05 (0.007–0.54) | 0.052 |
|                                       | No     | 49 (81.67) | 0.14 (0.003–1.25) | 0.01 (0.005–0.91) | 0.187 |
| Fever                                 | Hyperthermia | 46 (76.67) | 0.04 (0.005–1.09) | 0.06 | 0.03 (0.008–0.74) | 0.052 |
|                                       | Hypothermia | 8 (13.33)  | 0.04 (0.001–0.22) | 0.04 (0.01–0.69) | 0.187 |
| Abdominal Distention                  | Yes    | 11 (18.33) | 0.01 (0.006–0.88) | 0.192 | 0.11 (0.07–0.93) | 0.03 |
|                                       | No     | 49 (81.67) | 0.04 (0.009–1.07) | 0.02 (0.07–0.86) | 0.04 |
| Respiratory distress                  | Yes    | 11 (18.34) | 0.05 (0.03–0.75)  | 0.206 | 0.03 (0.005–0.36) | 0.308 |
|                                       | No     | 49 (81.67) | 0.01 (0.006–0.07) | 0.03 (0.002–0.19) | 0.052 |
| 1                                     | 27 (45.00) | 0.01 (0.007–0.56) | 0.03 (0.007–0.82) | 0.27 |
| 2                                     | 12 (20.00) | 0.04 (0.006–0.88) | 0.1 (0.005–0.27) | 0.094 |
| 3                                     | 5 (8.33)  | 0.01 (0.002–0.79) | 0.03 (0.01–0.38) | 0.052 |
| 4                                     | 5 (8.33)  | 0.1 (0.004–0.58)  | 0.2 (0.004–1.07) | 0.087 |
| Bleeding                              | Yes    | 15 (25.0)  | 0.1 (0.006–1.03)  | 0.197 | 0.08 (0.01–0.83) | 0.094 |
|                                       | No     | 45 (75.0%) | 0.02 (0.006–0.98) | 0.01 (0.007–0.82) | 0.227 |
| Brady Cardia                          | Yes    | 4 (6.67)   | 0.2 (0.007–0.86)  | 0.176 | 0.03 (0.009–0.44) | 0.094 |
|                                       | No     | 56 (93.33) | 0.1 (0.003–0.49)  | 0.02 (0.009–0.58) | 0.087 |
| Apnea                                 | Yes    | 8 (13.33)  | 0.2 (0.001–0.79)  | 0.443 | 0.3 (0.005–0.68) | 0.087 |
|                                       | No     | 52 (86.67) | 0.01 (0.008–0.95) | 0.07 (0.007–1.01) | 0.187 |
which released importin α3 

miR21-5p – (0.989 documented score for severity and predicted mortality of NS. Our results confirmed the diagnostic and prognostic values of two target genes and the utility of using these genes as predictors of mortality in NS.

The current study focused on the measurement of the serum expression levels and determination of the clinical importance of miR181b-5p and miR21-5p in newborns with sepsis and explored the fold changes of miR181b-5p and miR21-5p in survivors versus non-survivors. Also, this study aimed at the declaration of diagnostic values of the target two genes and the utility of using these genes as predictors of mortality in NS.

In the present study comparison of demographic data between the NS group and controls demonstrated that there were many risk factors in the patient’s group included; significant lower gestational age and hence a significantly higher percentage of prematurity, and a significantly higher percentage of PROM, also, lower birth weight was detected in NS group with nonsignificant value (P = 0.59). All these risk factors were reported in previous studies [20].

In this study, real-time-PCR showed that the serum expression levels of miR181b-5p and miR21-5p were significantly downregulated in newborns with sepsis compared with those in the controls, which were of diagnostic value with considerable sensitivity and specificity.

Up to our knowledge, no other study measured the serum miR181b-5p in NS, so we will discuss our results against adult sepsis patients. Our findings were consistent with Sun et al., 2012 [7] who reported that intensive care unit patients with sepsis had decreased levels of miR-181b compared with control individuals, and injection of mice with proinflammatory stimuli reduced miR-181b expression. Similarly, Yang et al., 2021 [10] reported reduced miR-181b in cultured neonatal cardiomyocytes treated with lipopolysaccharides (LPS), and the induction of its expression suppresses the production of proinflammatory cytokines induced by LPS in vitro.

Regarding miR-21, Na et al., 2020 [6] documented that miR-21 expression was lower in sepsis patients when compared to healthy controls. Also, Zou et al., 2021 [21] reported that miR-21 level in T lymphocytes in septic rats was decreased than those of normal control rats and the expression of miR-21 was negatively correlated with inflammatory factors.

Paradoxically, in a septic rat model McClure et al., 2014 [12] found that miR-21 and miR-181b expressions were high in early sepsis and continued throughout late sepsis with a robust positive association with the immunosuppressive state in late sepsis and with higher mortality. Also Zhao et al., 2020 [22] hypothesized that significantly elevated miR-21 may also be involved in the progression of the disease in rats with induced sepsis. Moreover, recent two studies concluded that upregulated circulating miR-21 was detected in neonatal sepsis patients compared to healthy neonates [23,24].

The ROC curve examination exhibited that miR21-5p had the highest AUC (0.917), followed by miR181b-5p (0.833), and with cutoff points equal (0.067) for miR21-5p and equals (0.324) for miR181b-5p, both

9. Predicted target genes of miR181b-5p and miR21-5p in neonatal sepsis

We designated Fig. 5 to show the predicted target genes and the suggested role of miR181-5p and miR21-5p in proinflammatory and immunosuppressive stages of neonatal sepsis. In the pro-inflammatory state of sepsis, both miRNAs decreased which released importin α3 [4] and PDCD4 [9] from inhibition leading to activation of the NF-κB pathway and increased production of proinflammatory cytokines as well as decreased production of anti-inflammatory cytokines resulting in enhancement of vascular inflammation. With the aggravation of inflammatory reactions, the body enters a state of immunosuppression, both miRNAs increased and were associated with a significant rise in the anti-inflammatory cytokines that override the immune system and paralyze it. Also, increased both miRNAs resulted in increased MDSCs cells which have strong suppressant effects on innate and adaptive immunity [12].

5. Discussion

The sepsis inflammatory response is a complicated biological and pathophysiological process that represents a struggle between proinflammatory and anti-inflammatory mediators initiated by exposure to infectious organisms. The sepsis initial exaggerated inflammatory reaction is characterized by the hyper-production of pro-inflammatory mediators that if not managed appropriately, change to an extended immunosuppressive state with hyperactive anti-inflammatory cytokines that amends both innate and adaptive immunity and is associated with high mortality [4,19].

Depending on the abovementioned facts, early sepsis diagnosis and management is a life-threatening procedure. Measurement of circulating miRNAs that exist in the blood is an easy and accessible clinical test that aid in early detection and consequently rapid correction of this serious syndrome. Collecting evidence specifies a crucial role for the miR181b-5p and miR21-5p in sepsis via affecting essential signaling pathways, for instance, the NF-κB pathway [4].

Table 6

| Age of onset (N) | Mean ± SD | Median | IQR | P value |
|------------------|-----------|--------|-----|--------|
| miR181b-5p       |           |        |     |        |
| Day1–3 (26)      | 0.676 ± 1.041 | 0.13 | 0.0099–0.401 | 0.452 |
| > day 3 (34)     | 0.508 ± 0.674 | 0.34 | 0.08–4.22 | 0.289 |
| miR21-5p         |           |        |     |        |
| Day1–3(26)       | 0.662 ± 1.656 | 0.16 | 0.007–7.16 | 0.132 |
| > day 3 (34)     | 0.213 ± 0.400 | 0.02 | 0.005–5.11 | 0.459 |

According to the age of onset

Table 7

| Variable          | Survivors (n = 38) | Non-Survivors (n = 22) | P value |
|-------------------|--------------------|------------------------|--------|
| miR181b-5p        | Mean ± SD          | Median (IQR)           |        |
|                   | 0.143 ± 0.025      | 0.099 (0.099–0.153)    | 0.0004 |
| miR21-5p          | Mean ± SD          | Median (IQR)           |        |
|                   | 0.054 ± 0.008      | 0.004 (0.004–0.09)     | 0.007  |
| SNAP II score (N) | Mean ± SD          | Median (IQR)           |        |
|                   | 9.45 ± 1.22        | 8.5 (5.5–15)           | < 0.001 |
| CRP               | Mean ± SD          | Median (IQR)           |        |
|                   | 21.68 ± 2.28       | 22 (11.2–24)           | < 0.001 |

According to SNAP II Score

prognostic values of target genes altogether with SNAP II which is the documented score for severity and predicted mortality of NS. Our results documented that compared to SNAP II score with AUC = 0.997 (0.989–1.00) with total accuracy 100%, miR181b-5p AUC was 0.737 (0.592–0.883) and accuracy exceeds 99% followed by miR21-5p with AUC was 0.680 (0.536–0.824) and accuracy reached 96%. These results confirmed the diagnostic and prognostic values of miR181b-5p and miR21-5p (Table 10, Fig. 4).

AUC (0.917), followed by miR181b-5p (0.997) with total accuracy 100%. miR181b-5p and miR21-5p in survivors versus non-survivors. Also, this study aimed at the declaration of diagnostic values of the target two genes and the utility of using these genes as predictors of mortality in NS.

Also, Zou et al., 2021 [21] reported that miR-21 level in T lymphocytes in septic rats was decreased than those of normal control rats and the expression of miR-21 was negatively correlated with inflammatory factors.

Paradoxically, in a septic rat model McClure et al., 2014 [12] found that miR-21 and miR-181b expressions were high in early sepsis and continued throughout late sepsis with a robust positive association with the immunosuppressive state in late sepsis and with higher mortality. Also Zhao et al., 2020 [22] hypothesized that significantly elevated miR-21 may also be involved in the progression of the disease in rats with induced sepsis. Moreover, recent two studies concluded that upregulated circulating miR-21 was detected in neonatal sepsis patients compared to healthy neonates [23,24].

The ROC curve examination exhibited that miR21-5p had the highest AUC (0.917), followed by miR181b-5p (0.833), and with cutoff points equal (0.067) for miR21-5p and equals (0.324) for miR181b-5p, both
miRNAs yielded a high specificity, and sensitivity. and total accuracy reaches 96%. Depending on these findings, miR21-5p and miR181b-5p showed good performance for the prediction of NS accordingly, miR21-5p and miR181b-5p might be used as novel diagnostic biomarkers for NS.

Previous results could be explained by the target microRNA roles reported in the previous literature; both miRNAs were reported to be anti-inflammatory miRNAs [4,7]. It was documented that miR-181b negatively regulates NF-κB–mediated vascular inflammation by targeting importin-α3, a protein critical for NF-κB activation. Moreover, miR-181b also suppresses the expression of a subset of NF-κB target genes linked to inflammation such as adhesion molecules (e.g., VCAM-1, E-selectin), chemokines, and chemokine receptors (CCL1, CCL7, CX3CL1, CXCL1, CCR2), and other important inflammatory mediators (e.g., COX-2, PAI-1) [7]. In addition, experimental models have shown that in response to LPS, miR-181 was downregulated, and in turn, this knocking down effect was associated with increased production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, and IL-8 [4].

Other members of the miR-181 family have been proven to have a role in regulating vascular inflammation and immunity [25], however, no study was done on NS except which done by Liu et al, 2019 who found that levels of miR-181a in serum were lower in neonatal sepsis patients.

Fig. 3. Dot plot distribution curves of fold change in miR181b-5p (A & B) and miR21-5p (C & D), regarding survival in the NS group; (A) Relative expression of the fold change of the serum miR181b-5p in survivors. (B) Relative expression of the fold change of the serum miR181b-5p in non-survivors. (C) Relative expression of the fold change of the serum miR21-5p in survivors. (D) Relative expression of the fold change of the serum miR21-5p in non-survivors.

Table 8
Univariate logistic regression analysis to detect independent predictors of septic mortality.

| variable       | OR    | Exp (B) | CI     | P value |
|----------------|-------|---------|--------|---------|
| age            | 0.020 | 0.994–1.258 | 0.629 |         |
| Gestational age| 0.042 | 1.068   | 0.026–1.478 | 0.187  |
| gender         | −0.452 | 0.696 | 0.773–11.254 | 0.636  |
| CRP            | 0.021 | 0.979   | 0.0258–5.478 | 0.032  |
| TLC            | 0.00017 | 1.00 | 0.987–6.358 | 0.010  |
| Lymphocytic count | 0.00043 | 1.00 | 0.0278–3.295 | 0.068  |
| CO2            | 0.021 | 0.980   | 0.856–1.758 | 0.397  |
| HCO3           | 0.044 | 1.045   | 0.415–3.927 | 0.358  |
| SNAP II score  | 0.987 | 0.667   | 0.338–2.566 | 0.010  |
| miR181b-5p     | 1.015 | 2.759   | 0.1986–5.0864 | 0.021  |
| miR21-5p       | 2.231 | 9.311   | 0.0148–7.0258 | 0.038  |

Table 9
Multivariate logistic regression analysis to detect independent predictors of septic mortality.

| Variable       | B     | S.E.  | P     | 95% CI for B |
|----------------|-------|-------|-------|--------------|
| miR181b-5p     | 2.345 | 0.985 | 0.012* | 0.487–5.0864 |
| miR21-5p       | 9.25  | 0.125 | 0.05*  | 0.125–3.087  |
| SNAP II score  | −0.366 | 0.054 | 0.08*  | 5.88–29.87   |
| CRP            | 4.135 | 0.229 | 0.097  | 1.0974       |
| TLC            | 0.012 | 0.101 | 0.0074 | 1.009        |
| Constant       | 7.338 | 3.854 | 0.024  | 9.258        |

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than in healthy neonates, and mir-181a upregulation repressed TLR4 representation in monocytes, LPS-induced inflammatory response, and the production of proinflammatory cytokines [26].

Regarding mir-21, researchers have found that mir-21 has a critical role in the resolution of inflammation by inhibiting the actions of PDCD4 on NF-kB activity and by inducing the production of anti-inflammatory cytokines [9]. Besides, mir-21 is a negative modulator of TLR4 of pro-inflammatory cytokines in LPS-stimulated macrophages as well as in response to infection, it inhibits the release of inflammatory mediators such as TNF-α, IL-6, and IL-12p40 [4,27].

Our results demonstrated that both genes are strongly positively correlated to each other with $r = 0.718$ and $p < 0.001$ which enforces their synergistic effects, while, are negatively correlated to CRP, leucocytes count, and lymphocyte count which are inflammatory markers, and these negative correlations reflect the anti-inflammatory action of both genes mentioned in the previous paragraph. Our results were consistent with Yang et al., 2021, and Li et al., 2021 [10, 28] who reported that mir-181b showed a significant negative association with the white blood cell count, absolute neutrophils, and the C-reactive protein of patients who suffered from inflammation with heart failure and from severe pneumonic patients respectively [10,28]. Also, Na et al., 2019 reported that mir-21 was negatively correlated with CRP in a patient with sepsis [6]. Also, this study’s results showed that there was a significantly positive correlation between serum mir181b-5p and RBS, this finding could be explained by the previous two studies done by Sun et al., 2016, and Wang et al., 2018 who reported that mir181b improves glucose homeostasis and insulin sensitivity by regulating endothelial function in white adipose tissue, and upregulation of mir-181b-5p reversed high-glucose-induced suppression of glycogenesis by targeting early growth response 1 (EGR1) [29,30]. Hence, increased mir181b-5p in response to increased RBS may be an attempt to regulate blood sugar which is an adverse factor in inflammation and sepsis.

Sepsis prognosis is just as crucial as the diagnosis and can impact therapeutic decisions and the known prognostic markers such as CRP and PCT remained with unsatisfactory specificities and sensitivities [31]. So, we followed newborns until they were released or died, whichever came first, up to a maximum of 20 days. Then, were further divided into survivors and non-survivors and explored whether target genes can be used as mortality predictors or not.

Our results demonstrated that the relative expression levels of mir181b-5p and mir21-5p were significantly overexpressed in non-survivors than survivors. Additionally, mir181b-5p and mir21-5p had an AUC of 0.737, 0.680 and yielded 99.8, 95.4% specificity and 98.9, 96.6% sensitivity when the cutoff point was set at 0.094, 0.059 respectively when ROC curve analysis was achieved to predict the outcome during sepsis. Also, ROC curve analysis of combined genes (mir181b-5p and mir21-5p) increases the sensitivity and total accuracy of mir181b-5p to reach 100%.

**Table 10**

| Variable         | AUC (95% CI) | Cut off point | P-value | sensitivity | Specificity | Total accuracy |
|------------------|-------------|---------------|---------|-------------|-------------|----------------|
| mir181b-5p       | 0.833 (0.739–0.928) | 0.324         | < 0.001 | 91.5%       | 100%        | 95.75          |
| mir21-5p         | 0.917 (0.847–0.987) | 0.067         | < 0.001 | 92.40%      | 100%        | 96.2           |
| **Between Survivors and Non-Survivors** |            |               |         |             |             |                |
| mir181b-5p       | 0.737 (0.592–0.883) | 0.256         | 0.002   | 98.9%       | 99.8%       | 99.35          |
| mir21-5p         | 0.680 (0.536–0.824) | 0.094         | 0.021   | 96.6%       | 95.4%       | 96%            |
| Combined miRs    | 0.964 (0.914–1.00)  | 0.038         | < 0.001 | 100%        | 99.8%       | 99.9           |
| SNAP II          | 0.997 (0.989–1.00)  | 35.83         | < 0.001 | 100%        | 100%        | 100%           |

Fig. 4. ROC curve analysis to detect the diagnostic and prognostic performance of target genes altogether with SNAP II.
A: ROC curve analysis to elucidate the diagnostic values of the miRNAs. The area under curve (AUC) was 0.917 (0.847–0.987) for mir21-5p and 0.833 (0.739–0.928) for mir181b-5p with cutoff = 0.067 and 0.324 respectively with total accuracy reaches nearly 96% for each.
B: ROC curve analysis was created to reveal the prognostic values of target genes altogether with SNAP II which is the documented score for severity and predicted mortality of NS. Our results documented that compared to SNAP II score AUC = 0.997 (0.989–1.00) with total accuracy 100%, mir181b-5p AUC was 0.737 (0.592–0.883) and accuracy exceeds 99% followed by mir21-5p with AUC was 0.680 (0.536–0.824) and accuracy reached 96%. ROC curve analysis of combined genes (mir181b-5p and mir21-5p) increases the sensitivity and total accuracy of mir181b-5p to reach 100%.
score, these results enforce the prognostic values of miR181b-5p and miR21-5p. Because sepsis is a complex illness involving several organs and tissues, a single biomarker is insufficient to reflect either the predictive potential for sepsis prognosis or severity, our genes could be paired with SNAP II for increased precision. Consistent with our results, McClure et al., 2014 [12] documented that miR-21 and miR-181b inductions in myeloid progenitors, leading to increased accumulated septic myeloid-derived suppressor cells (MDSCs). MDSCs have a significant immunosuppressive effect that suppresses both innate and adaptive immune responses, resulting in fatal secondary infections, while antagonists blockage of miR-21 and miR-181b in vivo, improving late-sepsis survival in mice [12]. Also, miR-21 overexpression in rats was linked to a poor prognosis and a lower survival rate, according to Zhao et al., 2020 [22]. Furthermore, the results of Xue et al., 2019 [33] demonstrated that miR-21 knockout mice died at a considerably lower rate than wild-type mice after peritoneal injection of LPS. Moreover, Salim et al., 2020 [23] reported significantly upregulated miR-21 in non-survivors vs, survivors in neonatal septic patients. On contrary, de Melo et al., 2021 [11] discovered that sepsis increased miR-21 expression in peritoneal macrophages and neutrophils from septic C57BL/6J mice, and that deleting the miR-21 locus in myeloid cells enhanced animal survival. The discrepancy between these studies’ results might be because of different experimental settings or cell types used in each.

Above mentioned facts suggest that reduced target miRNAs seem to be in early sepsis to mediate activation of the NF-κB signaling pathway and consequently increase proinflammatory cytokine production. This hypothesis is consistent with Zou et al., 2021 [21], who documented that expression levels of miR-126 and miR-21 firstly decreased, then increased, and were negatively correlated with the TNF-α, IL-6, and caspase-3 release and activity. With the aggravation of inflammatory reactions, the body enters a state of immunosuppression in sepsis with high anti-inflammatory mediators thus both miRNAs increased in this immunosuppressive phase [12] and were associated with a significant rise in the anti-inflammatory cytokines that override the immune system and paralyze it to control tissue damage occurred during the early hyperinflammatory phase. But, consequently, the suppression of anti-inflammatory response increases secondary infection susceptibility [34] and is associated with increased mortality [9].

This research has minor limitations. The sample size of the current study was modest due to the serious illness of the newborns who were recruited. There is minor preceding research to link our findings to, and the sample size is somewhat small. Thus, further work is required on a larger sample size aiming at the prediction of mRNAs targeted by miR181b-5p and miR21-5p in neonatal sepsis-affected newborns from healthy neonates and their utility as diagnostic biomarkers for NS. Furthermore, based on multiple data not previously published in any earlier studies, this study was the first to highlight the usefulness of miR181b-5p and miR21-5p in predicting the prognosis of newborn sepsis.

6. Conclusion

The major findings of this study are the possibility of using miR181b-5p and miR21-5p to aid in the diagnosis of NS and predict the sequels of the disease.

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CRediT authorship contribution statement

Marwa A. Ali: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – original draft, Supervision. Sherin Khamsi Hussein: Resources, Data curation, Writing – review & editing. Esam Ali Mohamed: Methodology, Validation, Writing – original draft,
Supervision. Mostafa Ahmed Ezzat: Validation, Supervision. Abdelrahman Abdelmoktader: Methodology, Data curation. Marwa A. Habib: Formal analysis, Writing – review & editing. Marwa Kamal: Resources, Writing – review & editing. Fatma A. Ahmed: Methodology, Validation, Visualization. Doaa Y. Ali: Methodology, Investigation, Visualization.

Declaration of competing interest

The authors declare that they have no competing interests.

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