Diagnostic predictability of miR-4535 and miR-1915-5p expression in amniotic fluid for foetal morbidity of infection

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Abbreviations[1]

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AUC	Area under the curve
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
FIRS	Foetal inflammatory response syndrome
PPROM	Preterm premature rupture of membrane
ROC	Receiver-operating characteristic
RT	Reverse transcription
SIRS	Systemic inflammatory response syndrome
WBC	White blood cell

Abstract

Introduction: Clinical prediction of foetal inflammatory response syndrome (FIRS) is highly necessary. We have previously reported that miR-4535 and miR-1915-5p are potential biomarkers for severe chorioamnionitis based on the results of microRNA array analysis. Therefore, we evaluated the relationship between foetal morbidity of infection and miR-4535, miR-1915-5p, interleukin (IL)-6, or 16S rDNA copy number levels in amniotic fluid from pregnant women with chorioamnionitis.

Methods: Amniotic fluid from 57 pregnant women with preterm premature membrane rupture or threatened premature labour were collected. Infants with WBC counts <5,000/μL or >20,000/μL, CRP >0.5 mg/mL, or IgM >20 mg/mL at birth received a diagnosis of suspicious foetal infection, and those requiring antibiotic administration for >5 days were considered infected newborns. miR-4535, miR-1915-5p, and IL-6 levels and 16S rDNA copy number were evaluated. Mann-Whitney U test and Dunn's test were used for comparison. The area under the curve (AUC) and Youden index were calculated to examine the diagnostic accuracy of foetal morbidity of infection.

Results: miR-4535, miR-1915-5p, 16S rDNA, and IL-6 were significantly higher in patients with severe chorioamnionitis than in patients with chorionitis or sub-chorionitis ($P < 0.05$). miR-4535 and miR-1915-5p levels were significantly associated with WBC counts

<5,000/μL or >20,000/μL, CRP >0.5 mg/mL, or IgM >20 mg/mL ($P < 0.05$). AUC values of miR-4535 and miR-1915-5p indicated moderate or low accuracy for foetal morbidity of infection, while those of IL-6 and 16S rDNA seemed unreliable.

Discussion:

MiR-4535 and miR-1915-5p levels in amniotic fluid may be considered clinically predictive for foetal morbidity of infection.

Keywords: miR-4535, miR-1915-5p, interleukin-6, 16S rDNA, fetal infection

Highlights

- Specific miRNA levels were elevated in amniotic fluid in severe chorioamnionitis
- Inflammatory biomarkers and infectious index were significantly correlated
- An association was observed between specific miRNAs and sustained neonatal sepsis
- Specific miRNAs could be accurate markers for foetal morbidity of infection

Introduction

Some bacteria can infect and spread into the uterine cavity, leading to extensive cytokine production in the amniotic fluid [1,2], resulting in intra-amniotic inflammation [1,2]. Such intra-amniotic inflammation is mainly induced by enhancement of interleukin-6 (IL-6) in amniotic fluid, which is significantly associated with acute chorioamnionitis and funisitis [3,4]. Under intra-amniotic inflammatory conditions, foetuses are exposed to many cytokines, including IL-6, and rapidly show a hyper-cytokine storm called the foetal inflammatory response syndrome (FIRS) [5–7]. In particular, foetuses in preterm premature rupture of membranes have a high tendency to develop FIRS, including foetal infection [5–7]. Foetuses with FIRS have a higher frequency of severe neonatal morbidity associated with suspected or proven neonatal sepsis, lung disorders including respiratory distress syndrome, pneumonia, and bronchopulmonary dysplasia; brain damage including intraventricular haemorrhage, periventricular leukomalacia, and cerebral palsy; and necrotising enterocolitis [5,8]. Therefore, accurate prediction of FIRS before delivery is clinically required.

In principle, FIRS is a clinical state affected by systemic inflammation and is characterised by the upregulation of foetal plasma IL-6 (> 11 pg/mL) [5,8]. The haematologic response of foetuses with FIRS is also defined by significant changes in

the total white blood cell and neutrophil counts [7]. Therefore, to diagnose FIRS before delivery, the level of IL-6 and the count of WBCs in the umbilical cord need to be determined by cordocentesis. However, cordocentesis for any patient with intra-amniotic inflammation seems to be impossible as well as clinically dangerous. Easy-to-use amniocentesis has been adapted for patients with intra-amniotic inflammation to predict FIRS in the antepartum period [9,10]. In contrast, C-reactive protein (CRP), Il-6, and IgM have also been considered useful for predicting neonatal sepsis, similar to an abnormal WBC count [11, 12, 13]. Therefore, various cytokines and inflammation-associated substances in amniotic fluid have been determined to indirectly diagnose FIRS or neonatal sepsis in patients with intra-amniotic inflammation [4,6,9,10]. Among these, the level of IL-6 in amniotic fluid has been the focus for the prediction of foetal infection or FIRS [14,15]. Recently, through microRNA array analysis in amniotic fluid, the expression of miR-4535 and miR-1915-5p in amniotic fluid was reported as a promising biomarker for severe chorioamnionitis [16]. Such clinically valuable biomarkers should be investigated to anticipate intra-amniotic inflammation as well as foetal infection or FIRS.

MicroRNAs (miRNAs) are small non-coding RNAs (21–25 nt) that regulate the transcription of target genes [17–19]. miRNAs are involved in various cellular behaviours, including cell proliferation, differentiation, and death [17–19]. miRNAs exist in various

biological fluids, such as pulmonary, abdominal, and amniotic fluids, as well as in circulating body fluids such as plasma and serum, and in urine, semen, and menstrual blood, as nano-sized vesicular particles [20–22]. As a result, some miRNAs in bio-fluids have been proposed as ideal disease biomarker candidates [20–22]. In a variety of infectious diseases, the participation of miRNAs during infection mediated by various pathogens, including parasites, bacteria, and viruses, is a well-established concept [23–25]. Accumulating evidence indicates that many miRNAs regulate the complex interplay between bacterial survival strategies and the host innate immune system pathways [26,27]. Thus, it is plausible that alterations in host miRNAs are most informative as trace substances for chorioamnionitis.

To assess the clinical significance of novel biomarkers for foetal infection associated with FIRS, we examined the predictive values of IL-6, miR-4535, miR-1915-5p, and 16S rDNA levels for foetal morbidity of infection. First, we examined the relationship between the four biomarkers in amniotic fluid and inflammation in the placenta and the correlation among the four biomarkers in amniotic fluid. Second, we analysed the association between the aberrant inflammatory laboratory findings, including abnormal WBC count, high CRP and IgM, and the four biomarkers in amniotic fluid. Finally, we evaluated the

clinical significance of the four biomarkers in suspicious neonatal sepsis patients treated with long-term antibiotic administration.

2. Material and methods

Histological criteria

Histological chorioamnionitis was diagnosed based on findings of acute inflammatory sites in the chorion or amnion following Blanc's criteria [28]. In stage III (chorioamnionitis), neutrophilic infiltrate is found in sub-amniotic connective tissues as well as the amniotic epithelium; in stage II (chorionitis), neutrophilic infiltration is detected in the chorionic plate or the membranous chorionic connective tissues; in stage I (sub-chorionitis), patchy or diffused accumulation of neutrophils is observed in the sub-chorionic plate or decidua.

Clinical definition

A high white blood cell (WBC) count (>20,000) or low WBC count (<5,000) in peripheral blood are significantly associated with culture-proven neonatal sepsis [11,29]. The values of neonatal CRP and IgM were also clinically evaluated to investigate neonatal sepsis [13]. Considering the diagnostic significance of WBC counts in neonatal sepsis, in the present study, we defined suspicious foetal infection as WBC counts <5,000/μL or

>20,000/μL, CRP >0.5 mg/mL, or IgM >20 mg/mL in neonates after delivery. Non-foetal suspicious infection was defined as a WBC count ranging between 5,000/μL and 20,000/μL, CRP <0.5 mg/mL or IgM <20 mg/mL, which is also associated with early-onset neonatal sepsis [12,13].

Patients

Amniotic fluid samples were examined in 57 pregnant women, including 11 with Blanc's classification Stage III (group III), 20 Blanc's classification Stage II (group II), and 26 with Blanc's classification Stages 0–I (group I). All patients were diagnosed with preterm premature rupture of membrane (PPROM) or threatened premature labour and were admitted to the hospital and were suspected of having an intrauterine infection. Among the pregnant women with PPROM, 8 of 11 cases were in group III, 5 of 20 cases were in group II, and 3 of 26 cases were in group I. Further, 31 pregnant women in groups II and III, and 22 of 26 pregnant women in group I delivered within at least four days of amniocentesis. The remaining four pregnant women in group I, who delivered at almost two weeks after amniocentesis, showed no symptoms or signs of uterine infection until delivery. The clinical results of newborns were available for the 57 pregnant women in all groups. Peripheral blood was obtained from neonatal infants on their date of birth. Seventeen of the 57 infants received long-term administration of antibiotics for more than

5 days and received a diagnosis of suspicious neonatal sepsis. The Ethics Committee of Fukuoka University Hospital and the National Hospital Organisation Saga National Hospital approved this study (approval numbers 15-2-08 and 23–4). All participants provided written informed consent, and the potential risks were clearly explained to them. Clinical and pathological information for pregnant women was obtained from their clinical records.

Sample collection

Amniotic fluid samples from pregnant women were collected at Fukuoka University Hospital (*n* = 18) and National Hospital Organization Saga National Hospital (*n* = 39) from 2009 to 2019. To obtain amniotic samples, amniocentesis was performed percutaneously under transabdominal ultrasound guidance in sterile conditions. Peripheral blood was obtained from neonates after birth. After collecting amniotic fluid samples, they were centrifuged at 3,000 rpm for 20 min at 25 ± 1 °C. The pellets were then separated and removed. The supernatant was stored at -80 °C until used for RNA extraction, DNA extraction, or enzyme-linked immunosorbent assay (ELISA).

Total DNA and RNA extraction

Total RNA was isolated from 200 μL amniotic fluid using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany), and was spiked with commercially

synthesised *Caenorhabditis elegans* miR-39-3p (Cel-miR-39-3p; Genenet Co., Ltd, Fukuoka, Japan) as a control. Total RNA was purified according to the manufacturer's protocol and was eluted in 35 µL RNase-free water. DNA was extracted using the QIAamp UCP Pathogen Mini Kit (Qiagen, Hilden, Germany), as described previously [30].

Reverse transcription (RT) and digital droplet polymerase chain reaction (ddPCR)

For cDNA synthesis of miRNAs using a TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), 5 µL of total RNA was used for each RT reaction with specific primers for miRNAs and Cel-miR-39-3p, according to the manufacturer's protocol. miRNAs were quantified by droplet-based digital PCR (ddPCR) using TaqMan® miRNA Assays. The cDNA (2 µL) was diluted in 18 µL of Bio-Rad QX200 reagent (Bio-Rad, Hercules, CA, USA), and each sample was then partitioned into approximately 20,000 droplets using the QX200 Droplet Generator (Bio-Rad). PCR was performed using the cycling conditions recommended by the manufacturer's protocol (annealing temperature: 52 °C). TaqMan primers and probes for each miRNA were obtained from Applied Biosystems. For absolute quantification of 16S rDNA, ddPCR was conducted with EvaGreen dye using universal primers (27Fmod and 338R) (Sigma-Aldrich Japan, Tokyo, Japan) as described in a previous study [30].

Interleukin-6 (IL-6) ELISA

The level of IL-6 in amniotic fluid samples was measured by ELISA using high sensitivity kits (Quantikine®HS, R&D Systems Minneapolis, USA). Duplicate analyses were conducted in accordance with the manufacturer's instructions and the analysis protocol. Finally, the microtiter plate was read at 450 nm (correction wavelength 570 nm) using a microplate reader (SLT Spectra, Tecan). The results are presented as the mean ± standard deviation.

Statistical analysis

Clinical data were statistically analysed using the Mann-Whitney U test and Chi-squared test. For ddPCR and ELISA, statistically significant differences in values were determined using the Mann-Whitney U test and Dunn's multiple comparisons test. All data are expressed as the mean ± standard error of the mean. The cut-off values of miR-4535, miR-1915-5p, 16S rDNA, and IL-6 were the medians of expression in amniotic fluid among 57 pregnant women. To evaluate diagnostic significance, we conducted receiver-operating characteristic (ROC) curves. We also calculated the area under the curve (AUC) and Youden index and used Fisher's exact test. Correlation is presented as the Spearman correlation coefficient (r). Statistical significance was set at $P < 0.05$. Statistical analyses were performed using GraphPad Prism v8.0 (GraphPad Software, La Jolla, CA) and SPSS v16.0J (SPSS Japan, Tokyo, Japan) for Windows.

Results

Assessment of study subjects

Demographic and clinical characteristics of groups III, II, and I were extracted from the respective medical records (Table 1). Group III included a high proportion of pregnant women with premature rupture of membranes ($P < 0.05$). Maternal WBC count and maternal C-reactive protein (CRP) were significantly elevated in group III, compared to those in group I ($P < 0.05$). In addition, neonatal CRP and IgM in group III were significantly higher than those in group I ($P < 0.05$), whereas only neonatal CRP was significantly elevated in group III compared to group II ($P < 0.05$). Group III had significantly higher cases with an abnormal WBC count than groups I and II ($P < 0.05$). These results indicated that maternal and neonatal inflammatory responses to infection were enhanced in group III.

Clinical significance of miRNAs, 16S rDNA, and IL-6 for aberrant inflammatory findings

To confirm the clinical significance of the expression of miRNAs, IL-6, or the 16S rDNA copy number in chorioamnionitis, we examined these factors in all three groups.

The levels of miR-4535, miR-1915-5p, and IL-6 in group III were significantly upregulated compared to those in group I ($P < 0.05$) (Supplementary Figure 1). The copy number of 16S rDNA in group III was significantly higher than that in groups I and II ($P < 0.05$) (Supplementary Figure 1). The expression of miR-4535 and miR-1915-5p and the copy number of 16S rDNA were elevated in severe chorioamnionitis cases, similar to that observed in a previous study (13). The levels of miR-4535, miR-1915-5p, and IL-6 were significantly correlated with the 16S rDNA copy number ($P < 0.05$) (Figure 1). These results suggest that the expression of miR-4535, miR-1915-5p, and IL-6 may be dependent on the extent of placental infection, and the copy number of 16S rDNA may be directly associated with placental inflammation in patients with rupture of membrane.

To investigate the relationship between neonatal inflammation and the four biomarkers in amniotic fluid, we compared the level of miR-4535, miR-1915-5p, IL-6, and the 16S rDNA copy number in the absence or presence of abnormal findings, including neonatal WBC counts and CRP or IgM levels among all groups. In neonates with abnormal WBC count harbouring >20,000 cells/μL or <5,000 cells/μL, only the level of miR-4535 or miR-1915-5p was significantly upregulated, compared to that in infants with normal WBC count, ranging from 5,000 to 20,000 cells/μL ($P < 0.05$) (Table 2). However, no significant difference in the IL-6 level or 16S rDNA copy number was observed in

neonates between abnormal and normal WBC count groups (Table 2). In infants with more than 0.5 mg/mL CRP, all the biomarkers including miR-4535, miR-1915-5p, 16S rDNA, and IL-6 were significantly enhanced, compared to those in neonates with CRP levels below 0.5 mg/mL ($P < 0.05$) (Table 2). As for the neonatal IgM, in neonates with levels (≥ 20 mg/mL) of IgM, the levels of miR-4535 and miR-1915-5p, and the 16S rDNA copy number were significantly elevated, compared to those in infants with low levels (<20 mg/mL) of IgM (all $P < 0.05$) (Table 2). However, no significant differences in IL-6 levels were observed between neonates with high and low IgM levels (Table 2). According to the AUC analysis, miR-4535 and miR-1915-5p indicated more than moderate accuracy in predicting an abnormal WBC count and high CRP or IgM, whereas 16S rDNA and IL-6 revealed high accuracy only in predicting high CRP levels. Based on these lines of evidence, miR-4535 and miR-1915-5p may be considered promising biomarkers commonly associated with aberrant inflammatory findings, including abnormal WBC counts, high CRP, and IgM (Supplementary Table 1).

Diagnostic significance of miRNA levels

To predict FIRS based on the level of miR-4535, miR-1915-5p, or IL-6, and the 16S rDNA copy number in amniotic fluid, we calculated the AUC, Youden index, cut-off value, sensitivity, and specificity for the ROC curve. Using the ROC curve to examine the

diagnostic accuracy for patients with abnormal WBC count, the AUC values of miR-4535 and miR-1915-5p were found to be higher than those of 16S rDNA and IL-6 (Figure 2 and Supplementary Table 1). Using the ROC curve to examine the diagnostic accuracy for patients with CRP >0.5 mg/mL or IgM >20 mg/mL, each of the four biomarkers indicated moderate or high accuracy (Figure 2 and Supplementary Table 1). In amniotic fluid, detection of >1082.9 copies/μL of miR-4535 or >181.5 copies/μL of miR-1915-5p should provide important information on neonates harbouring abnormal WBC count, CRP >0.5 mg/mL, and IgM >20 mg/mL before delivery (Supplementary Table 1). Taken together, miR-4535 and miR-1915-5p in amniotic fluid seem to be superior to IL-6 as promising biomarkers for predicting aberrant inflammation, including abnormal WBC count and high CRP and IgM before delivery.

Relationship between aberrant inflammatory findings (WBC, CRP, IgM) or the four biomarkers and foetal morbidity of infection

In this study, 23 infants showed aberrant inflammatory laboratory findings, including abnormal WBC counts (Supplementary Figure 2A) and high CRP or IgM, and 17 infants were treated with long-term administration of antibiotics. Twelve of these 17 infants showed aberrant inflammatory findings, whereas the remaining five did not (Supplementary Figure 2B). The aberrant inflammatory findings, including high CRP and

IgM, were significantly associated with long-term administration of antibiotics, and abnormal WBC counts tended to be associated with medical intervention (Supplementary Table 2). The expression levels of miR-4535 and miR-1915-5p in infants with medical intervention were significantly enhanced compared to those in infants without medical intervention. However, no significant differences in the values of IL-6 and the copy number of 16S rDNA were found between infants with and without medical intervention (Figure 3). To predict foetal morbidity of infection based on the levels of miR-4535, miR-1915-5p, or IL-6 and the 16S rDNA copy number in amniotic fluid, we calculated the AUC, Youden index, cut-off value, sensitivity, and specificity for the ROC curve. Using the ROC curve to examine the diagnostic accuracy for infants with medical intervention, the AUC value of miR-4535 and miR-1915-5p was found to be significant, while the accuracy of 16S rDNA or IL-6 was insignificant (Table 3). This indicated that the AUC value of miR-4535 or miR-1915-5p revealed moderate or low accuracy for foetal morbidity of infection.

Discussion

In this study, elevated levels of miR-4535, miR-1915-5p, and IL-6 and copy number of 16S rDNA in amniotic fluid were found in severe chorioamnionitis cases. A significant association was observed between the expression of miR-4335 or miR-1915-5p and aberrant inflammatory laboratory markers including WBC counts < 5,000/μL or > 20,000/μL, CRP > 0.5 mg/mL, or IgM > 20 mg/mL. In infants treated with long-term administration of antibiotics, the expression of miR-4535 and miR-1915-5p in amniotic fluid was significantly elevated compared to those in patients without long-term antibiotic treatment. The AUC values of miR-4535 and miR-1915-5p revealed moderate and low accuracy, respectively, for foetal morbidity of infection.

FIRS seems to affect foetuses harbouring susceptibility to inflammation mediated by bacterial infection and prematurity [31], resulting in approximately 50% of foetuses with PPROM being affected by FIRS [5,32]. Among patients with PPROM, the median value of IL-6 in amniotic fluids as well as in vaginal secretions derived from the amniotic fluid as samples obtained with non-invasive methods was approximately 10,000 pg/mL in FIRS [14,15]. In this study, the best cut-off value of IL-6 for predicting the aberrant inflammatory laboratory markers or foetal morbidity of infection was less than 10,000 pg/mL in the ROC curve of IL-6. Therefore, IL-6 levels > 10,000 pg/mL may indicate the predictive value of FIRS in patients with premature labour, both with and without

membrane rupture. Previously, we reported that high expression of miR-4535 and miR-1915-5p in amniotic fluid was significantly associated with suspicious sepsis in neonates [16]. In this study, the expression of miR-4535 and miR-1915-5p in amniotic fluid revealed diagnostic accuracy in predicting aberrant inflammatory laboratory markers or foetal morbidity of infection. Overall, 8 of 11 cases (72.7 %) had preterm premature rupture of membranes, and 6 of 11 (54.5 %) cases were delivered before 28 weeks of gestation. As prematurity is accompanied by an inflammatory environment, the enhanced expression of miR-4535 and miR-1915-5p in amniotic fluid can promote the predictability for foetal morbidity of infection.

IL-6 has a broad effect on the cellular immune system and displays hormone-like properties linked with vascular disorders, lipid metabolism, and insulin resistance; thus, IL-6 plays a pivotal role in mitochondrial activity, the neuroendocrine system, and neuropsychological behaviour [33–35]. In addition, global blockade of IL-6, which is thought to be beneficial in autoimmune diseases, conversely promotes susceptibility to bacterial infections [36]. TNF-α and IL-1β have been recognised as the major activators of IL-6 expression, which are produced by immune cells as well as stromal cells in tissues, resulting in IL-6 being considered as the final functional molecule in the cascade of pro-inflammatory cytokines [37,38]. Several miRNAs have been reported to be involved in the

IL-6 signalling pathway [39,40], but there have been no reports concerning the relationship between IL-6 and miR-4535 or miR-1915-5p. In this study, miR-4535 and miR-1915-5p expression were found to be significantly powerful indicators, compared to the IL-6 levels. These results suggest that miR-4535 or miR-1915-5p might be produced through almost the same expression mechanism as IL-6 or that IL-6 itself might directly or indirectly be linked with miR-4535 and miR-1915-5p expression.

As for bacterial pathogens, miRNAs have been recognised as an integral part of the host immune response to efficiently fight infection during autophagy [41,42]. Importantly, emerging evidence demonstrates that bacteria actively harness host miRNAs to enhance their pathogenicity [26,43]. We previously reported that chorioamnionitis, involving infection by 11 types of bacteria, was considered a single or mixed infection and that *Ureaplasma parvum*, *Gardnerella vaginalis*, and *Streptococcus anginosus* were the main inflammatory bacteria [30]. There have been no reports of any specific miRNAs being expressed upon infection with these bacteria. In systemic inflammatory response syndrome (SIRS), several recent studies have identified miR-122, miR-150, and miR-223 in serum as potentially powerful diagnostic and predictive biomarkers for sepsis [44]. However, the biological functions of these miRNAs remain unclear. The functions of miR-4535 and miR-1915-5p, which are associated with FIRS, are also unknown. Accordingly,

miRNAs that are predominantly expressed in SIRS or FIRS might primarily function as infection-protective molecules.

In this study, the expression of miR-4535 and miR-1915-5p was significantly associated with foetal morbidity of infection. There have been few informative reports concerning the relationship between these predictive markers and infant morbidity. Further, it remains unclear whether pro-inflammatory cytokines, including IL-6, can regulate the expression of miR-4535 and miR-1915-5p. Accordingly, further studies should clarify the clinical significance or biological properties of miR-4535 and miR-1915-5p.

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Disclosure Statement

The authors have no conflict of interest.

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TABLE 1

Maternal and neonatal characteristics in Blanc's classification groups

	(A) group III (*n* = 11)	(B) group II (*n* = 20)	(C) group I (*n* = 26)	*P*-value (A *vs*. C)	*P*-value (B *vs*. C)	*P*-value (A *vs*. B)
Maternal characteristics at amniocentesis						
Age (y) †	35.0 (25-42)	31.0 (20-43)	33.0 (21-40)	0.670	1.000	0.207
Caesarean section §	7 (58.3)	9 (47.4)	11 (42.3)	0.295	1.000	0.457
Multigravida §	8 (72.7)	12 (52.6)	13 (50.0)	0.284	0.560	0.697
Gestational age at amniocentesis (weeks) †	27.43 (23.9–40.6)	33.71 (24.3–41.7)	32.71 (18.6–39.7)	0.692	0.847	0.136
Preterm premature rupture of membranes §	8 (72.7)	5 (25.0)	3 (11.5)	<0.001	0.267	0.020
Body temperature >38.0 (°C) §	3 (27.2)	3(15.0)	3 (11.5)	0.335	1.000	0.638
Heart rate >100 (/min) §	3 (27.2)	5 (25.0)	6 (23.1)	1.000	1.000	1.000
Tenderness of the uterus §	0 (0.0)	0 (0.0)	0 (0.0)	1.000	1.000	1.000
Stench of vaginal discharge or amniotic fluid §	0 (0.0)	0 (0.0)	0 (0.0)	1.000	1.000	1.000
WBC count in maternal peripheral blood >15,000 (cells/μL) §	8 (72.7)	10 (50.0)	5 (19.2)	0.006	0.055	0.275
CRP in maternal peripheral blood (mg/dL) †	4.2 (0.3–10.5)	1.47 (0.1–14.5)	0.48 (0.1-8.2)	0.005	0.085	0.623
Neonatal characteristics after birth						
Gestational age at birth (weeks) †	28.71 (24.9–40.6)	35.00 (26.3–41.7)	34.71 (24.4–39.7)	0.496	1.000	0.315
Premature birth (<37 weeks) §	9 (81.8)	14 (70)	22 (84.6)	1.000	0.292	0.675
Premature birth (<34 weeks) §	7 (58.3)	9 (47.4)	11 (42.3)	0.295	1.000	0.457
Premature birth (< 28 weeks) §	3 (27.2)	3 (15.0)	1 (3.8)	0.070	0.302	0.638
Neonatal body weight (g) †	1219.0 (743–3,665)	2177.0 (777–4,134)	2158.0 (697–3,116)	0.378	1.000	0.338
Apgar score 1 min †	7.0 (3–8)	7.5 (4–9)	8.0 (3–8)	0.077	0.402	1.000

Apgar score 5 min †	9.0 (5–9)	9.0 (6–9)	9.0 (5–9)	1.000	0.579	1.000
Umbilical arterial pH †	7.358 (7.20–7.44)	7.346 (7.18–7.51)	7.316 (7.18–7.49)	1.000	0.311	0.554
WBC count in neonatal peripheral blood >20,000 or < 5,000 (cells/μL) §	7 (63.6)	4 (20.0)	5 (19.2)	0.0183	1.000	0.0232
CRP in neonatal peripheral blood (mg/dL) †	0.34 (0.0–3.3)	0.00 (0.0–0.6)	0.00 (0.0–6.5)	<0.001	1.000	0.001
IgM in neonatal peripheral blood (mg/dL) †	11.0 (4–54)	7.5 (4–17)	4.0 (4–410)	0.005	0.152	0.414

† data shown as median (range), § data shown as n (%). WBC; white blood cells, CRP; C-reactive protein, IgM; Immunoglobulin M

TABLE 2

Median miR-4535, miR-1915-5p, 16S rDNA, and IL-6 levels in amniotic fluid of pregnant women when classified by their neonatal white blood cell counts, CRP, and IgM after birth

Neonatal WBC count (cells/μL)	5,000 ≤ WBC ≤ 20,000 (*n* = 41)	>20,000 or <5,000 (*n* = 16)	95% CI of difference	*P*-value
miR-4535 (copies/μL)	350.6	1,075	378.3 to 2,165	<0.0001
miR-1915-5p (copies/μL)	86.1	270.6	61.65 to 480.0	0.0004
16S rDNA (copies/mL)	2,970	102,795	-110.0 to 4,992,300	0.0636
IL-6 (pg/mL)	1,454	14,224	-14.00 to 16,598	0.0748
Neonatal CRP (mg/dL)	<0.5 (*n* = 48)	≥0.5 (*n* = 9)	95% CI of difference	*P*-value
miR-4535 (copies/μL)	361.1	544.9	56.35 to 4,825	0.0261
miR-1915-5p (copies/μL)	87.6	132.9	6.025 to 583.5	0.0350
16S rDNA (copies/mL)	2,860	874,500	18,590 to 26,797,100	0.0018
IL-6 (pg/mL)	709	13,261	4,278 to 42,851	0.0078
Neonatal IgM (mg/dL)	< 0 (*n* = 52)	≥20 (*n* = 5)	95% CI of difference	*P*-value
miR-4535 (copies/μL)	363.7	6918	360.6 to 7,827	0.0022
miR-1915-5p (copies/μL)	91.3	1707	113.9 to 1,839	0.0011
16S rDNA (copies/mL)	3,190	7,942,000	18,260 to 15,024,130	0.0059
IL-6 (pg/mL)	2,450	13,261	-1,925 to 62,867	0.0673

WBC, white blood cell; CRP, C-reactive protein; IgM, immunoglobulin M

TABLE 3

Diagnostic accuracy for foetal morbidity of infection; long-term administration of antibiotics for the newborn.

	AUC	Cut-off value	Youden Index	Sensitivity	Specificity	*p*-value
miR-4535	0.737	443(copies/μL)	0.439	0.675	0.765	0.0050
miR-1915-5p	0.673	69(copies/μL)	0.307	0.425	0.882	0.0404
16S rDNA	0.649	287,650(copies/mL)	0.354	0.825	0.529	0.0766
IL-6	0.664	3,575(pg/mL)	0.340	0.575	0.765	0.0518

AUC, area under the curve

Supplementary TABLE 1

Diagnostic accuracy for abnormal white blood cell count (<5,000 or >20,000 cells/μL), CRP, and IgM after birth

	AUC	Cut-off value	Youden Index	Sensitivity	Specificity	*p*-value
WBC count <5,000 or >20,000 cells/μL						
miR-4535	0.845	688.9 (copies/μL)	0.652	0.750	0.902	<0.001
miR-1915-5p	0.793	181.5 (copies/μL)	0.566	0.688	0.878	0.001
16S rDNA	0.660	4,147,000 (copies/mL)	0.365	0.483	0.927	0.062
IL-6	0.653	9,395.5 (pg/mL)	0.371	0.688	0.683	0.074
Neonatal CRP >0.5 (mg/dL)						
miR-4535	0.734	402.8 (copies/μL)	0.451	0.889	0.562	0.027
miR-1915-5p	0.722	92.8 (copies/μL)	0.562	1.000	0.562	0.036
16S rDNA	0.817	287,650 (copies/mL)	0.590	0.778	0.812	0.003
IL-6	0.775	4,005.5 (pg/mL)	0.583	1.000	0.583	0.009
Neonatal IgM ≥20 (mg/dL)						
miR-4535	0.888	1,082.9 (copies/μL)	0.685	0.800	0.885	0.004
miR-1915-5p	0.908	150.7 (copies/μL)	0.731	1.000	0.731	0.003
16S rDNA	0.858	4,150,000 (copies/mL)	0.685	0.800	0.885	0.009
IL-6	0.750	4,903.5 (pg/mL)	0.577	1.000	0.577	0.067

AUC, area under the curve; WBC, white blood cell; CRP, C-reactive protein*; IgM*, immunoglobulin M

Supplementary TABLE 2

Association between antibiotics administered to newborn for ≥ 5 days and aberrant inflammation findings (neonatal WBC, CRP, and IgM) in amniotic fluid using Fisher's exact test.

	Antibiotics for newborn ≥5 days (*n*=17)	Antibiotics for newborn <5 days (*n*=40)	*p*-value
Neonatal WBC count (cells/μL)			
>20,000 or < 5,000	8	8	0.0545
5,000 ≤ WBC ≤20,000	9	32	
Neonatal CRP (mg/dL)			
≥0.5	7	2	0.0018
<0.5	10	38	
Neonatal IgM (mg/dL)			
≥20	4	1	0.0242
<20	13	39	

WBC, white blood cell; CRP, C-reactive protein; IgM, immunoglobulin M

FIGURE 1

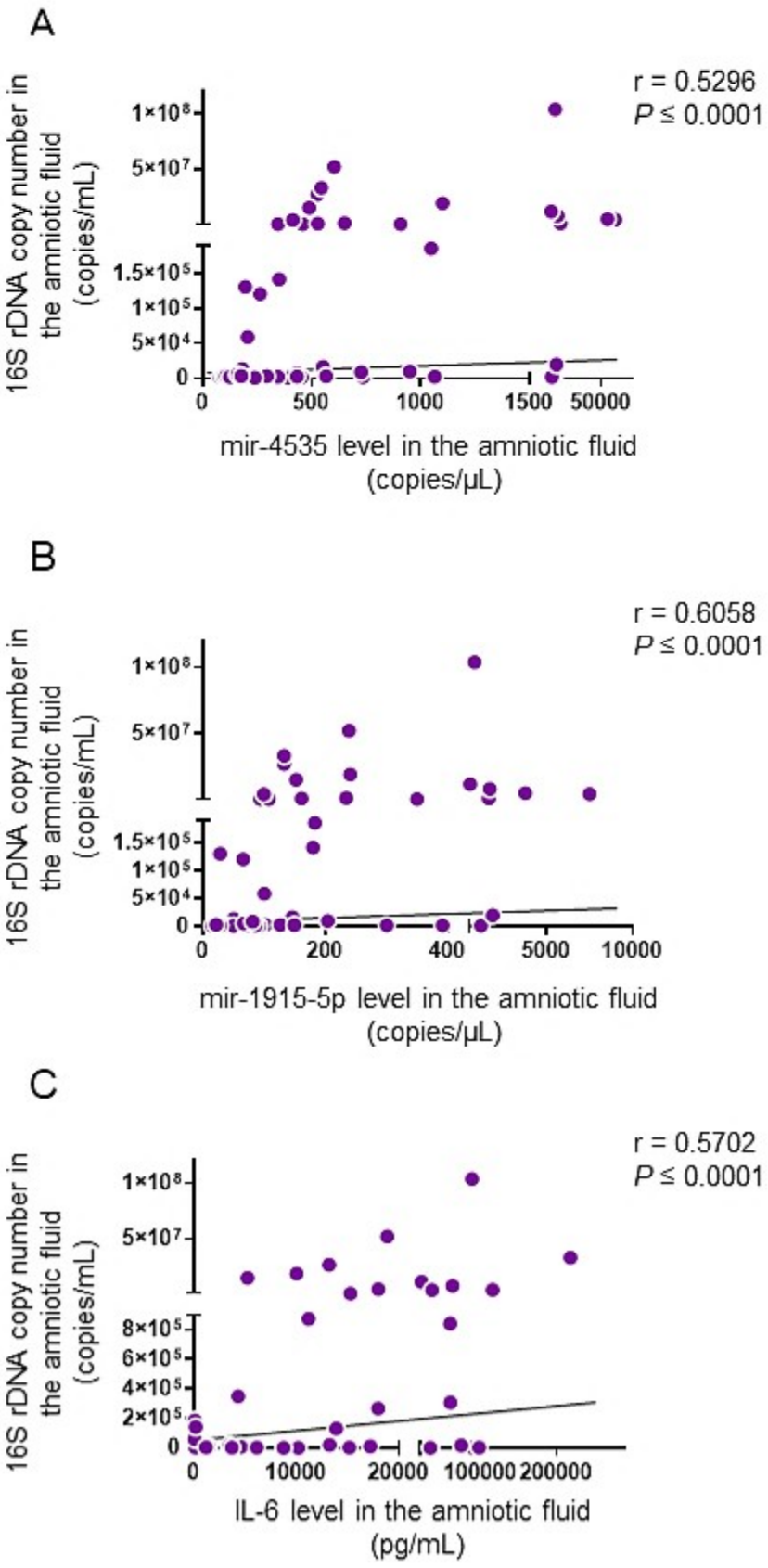
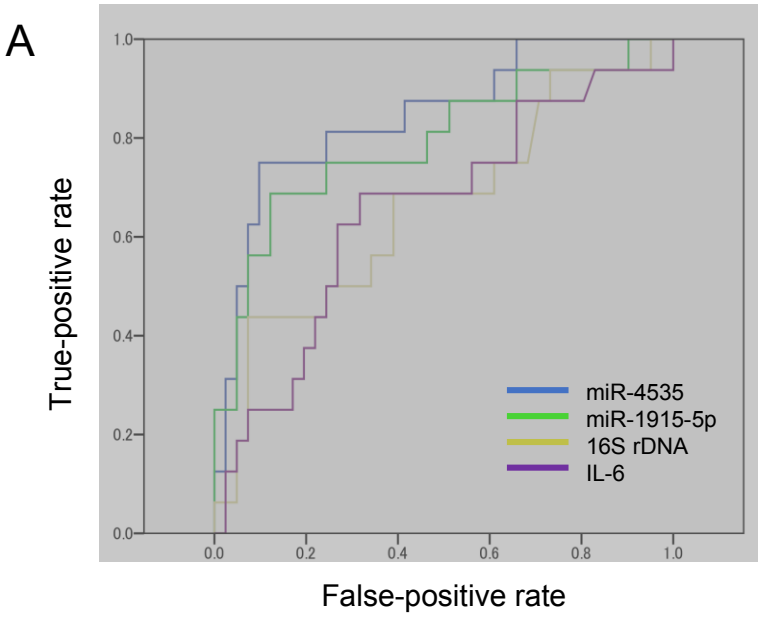
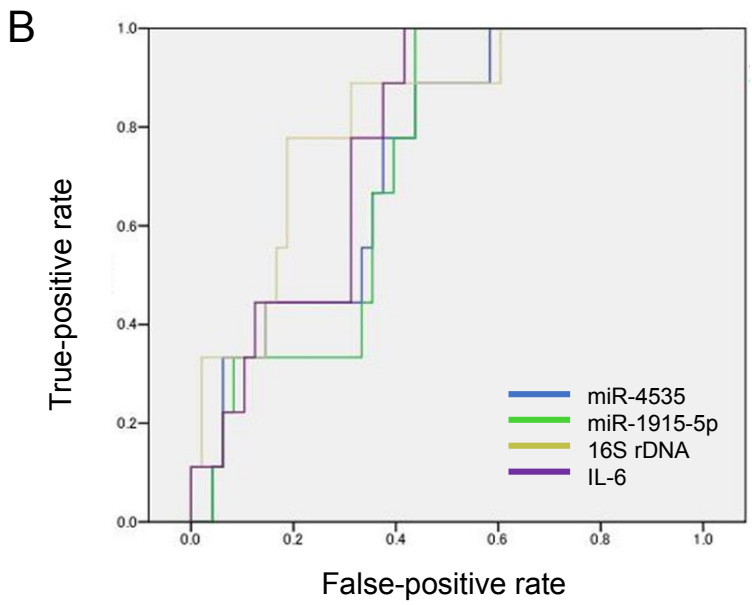

A
r = 0.5296
P ≤ 0.0001
16S rDNA copy number in the amniotic fluid (copies/mL)
1×10⁸
5×10⁷
1.5×10⁵
1×10⁵
5×10⁴
0
0
500
1000
1500
50000
mir-4535 level in the amniotic fluid (copies/μL)
B
r = 0.6058
P ≤ 0.0001
16S rDNA copy number in the amniotic fluid (copies/mL)
1×10⁸
5×10⁷
1.5×10⁵
1×10⁵
5×10⁴
0
0
200
400
5000
10000
mir-1915-5p level in the amniotic fluid (copies/μL)
C
r = 0.5702
P ≤ 0.0001
16S rDNA copy number in the amniotic fluid (copies/mL)
1×10⁸
5×10⁷
8×10⁵
6×10⁵
4×10⁵
2×10⁵
0
0
10000
20000
100000
200000
IL-6 level in the amniotic fluid (pg/mL)

FIGURE 2


A
True-positive rate
False-positive rate
miR-4535
miR-1915-5p
16S rDNA
IL-6


B
True-positive rate
False-positive rate
miR-4535
miR-1915-5p
16S rDNA
IL-6

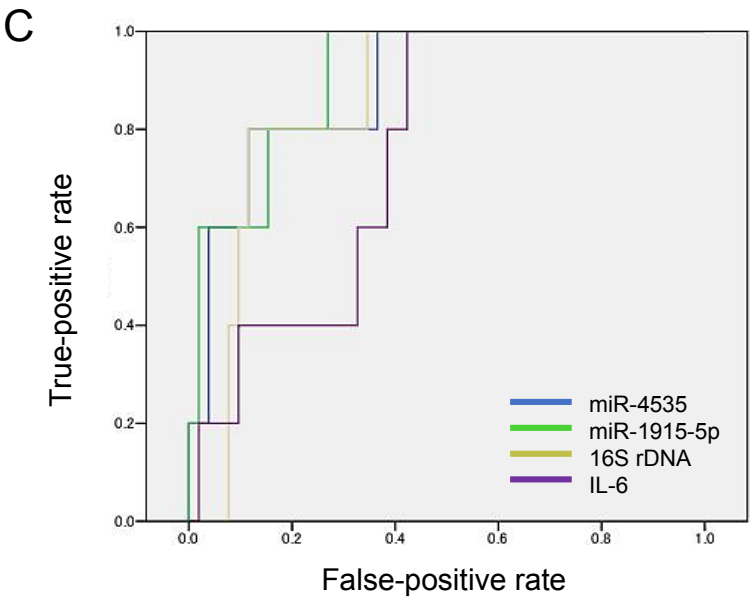
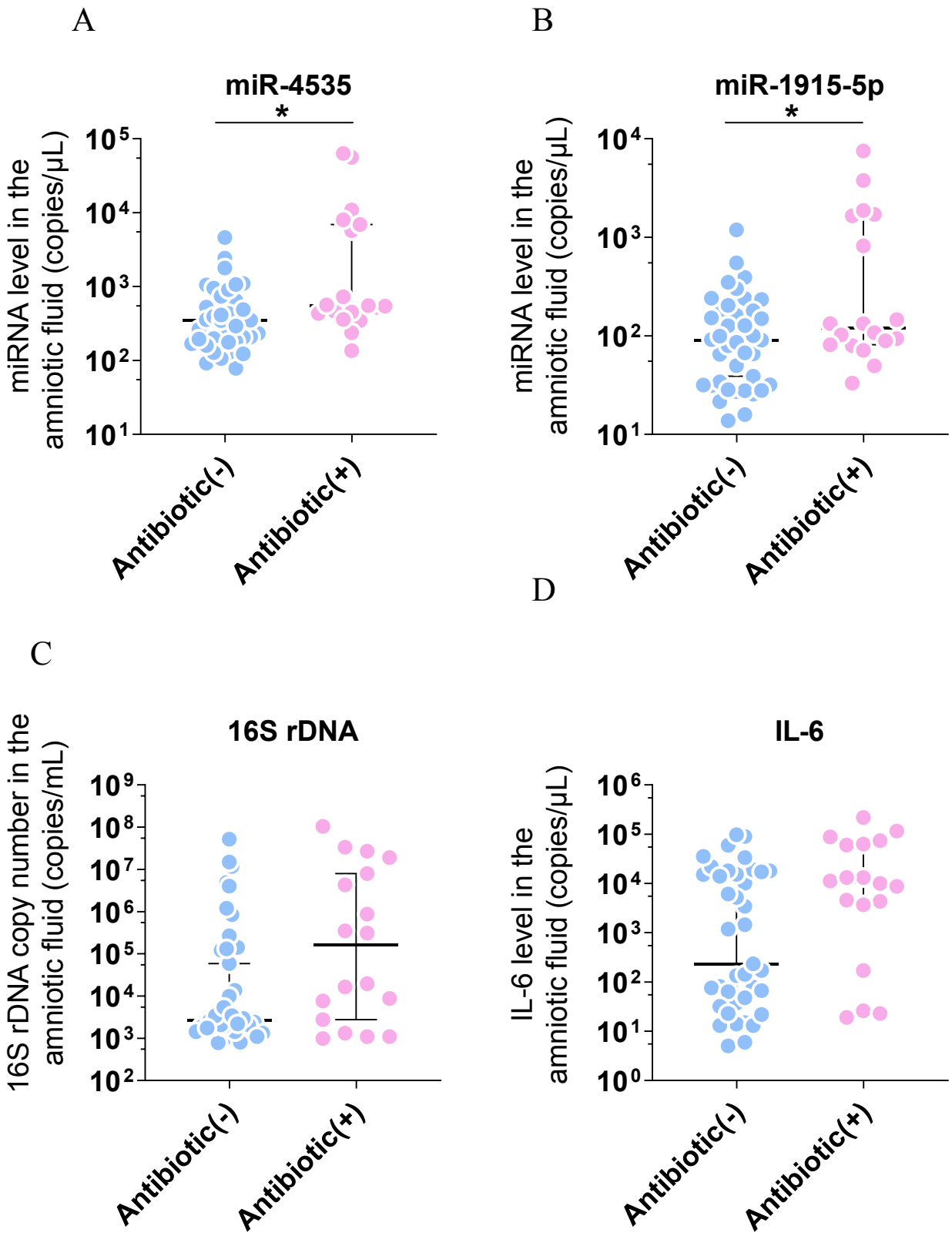
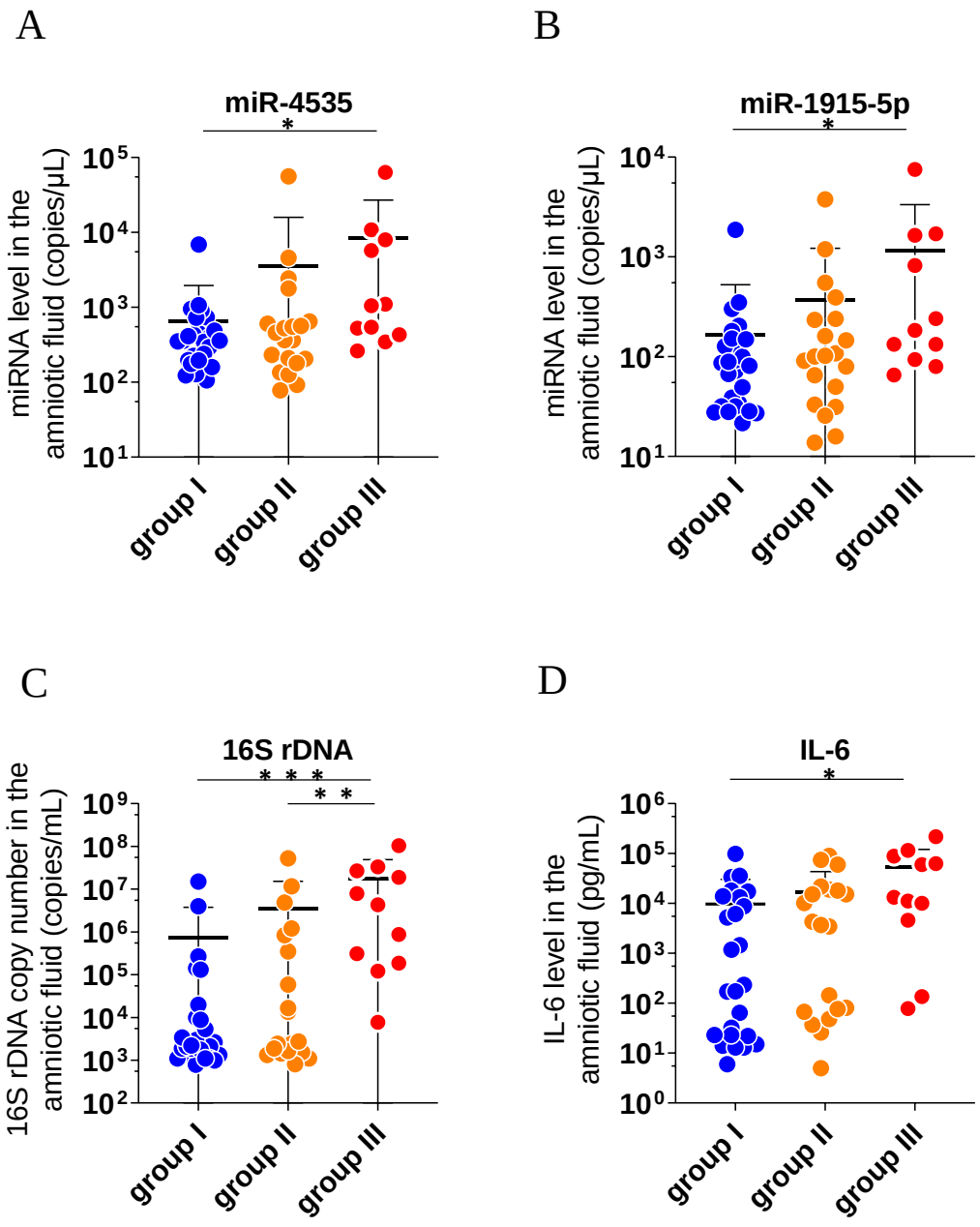

C
True-positive rate
False-positive rate
miR-4535
miR-1915-5p
16S rDNA
IL-6

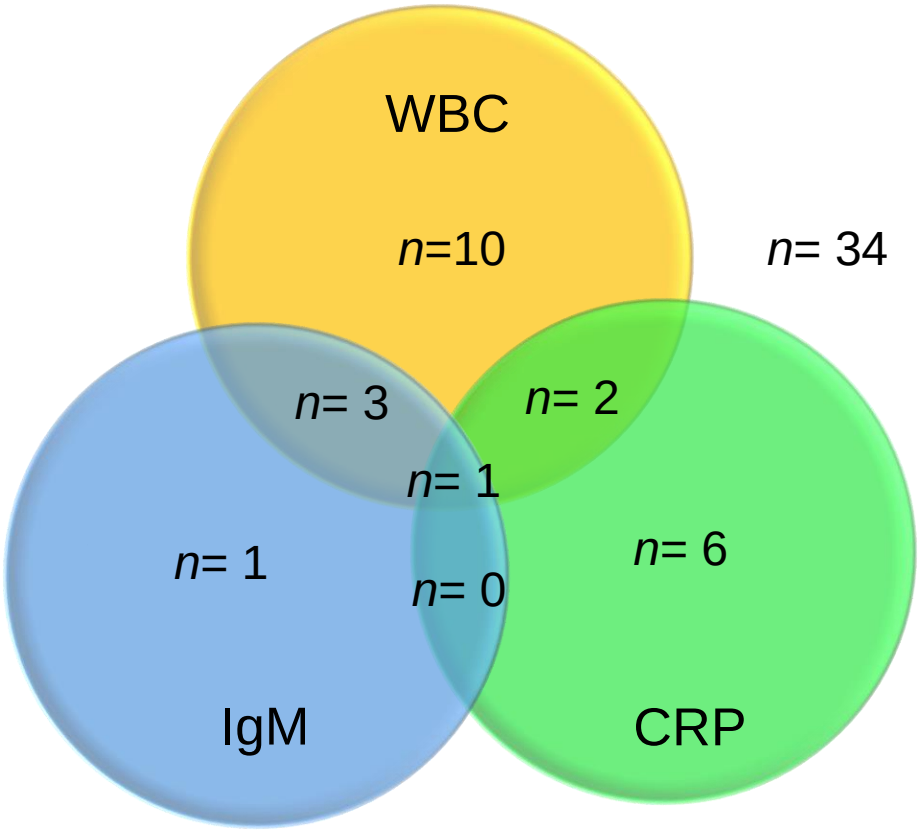
FIGURE 3


A
miR-4535
*
miRNA level in the amniotic fluid (copies/μL)
10^5
10^4
10^3
10^2
10^1
Antibiotic(-)
Antibiotic(+)
B
miR-1915-5p
*
miRNA level in the amniotic fluid (copies/μL)
10^4
10^3
10^2
10^1
Antibiotic(-)
Antibiotic(+)
C
16S rDNA
16S rDNA copy number in the amniotic fluid (copies/mL)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Antibiotic(-)
Antibiotic(+)
D
IL-6
IL-6 level in the amniotic fluid (copies/μL)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Antibiotic(-)
Antibiotic(+)

Supplementary FIGURE 1



Supplementary FIGURE 2A



Supplementary FIGURE 2B