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Basic Science: Obstetrics Nucleated Red Blood Cells are a Direct Response to Mediators of Inflammation in Newborns with Early Onset Neonatal Sepsis

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Abstract

Objective—To test the hypothesis that inflammation modulates fetal erythroblastosis and/or the release of NRBCs independent of hypoxia or fetal stress. We sought to determine if fetal inflammation is associated with an elevation in neonatal NRBC count in the setting of inflammation-associated preterm birth.

Study Design—The relationships between peripheral NRBC count, histological chorioamnionitis, umbilical cord interleukin-6 (IL-6), erythropoietin (EPO), cortisol and acid-base status were analyzed in 68 preterm singletons, born to mothers who had an amniocentesis to rule out infection. Proteomic profiling of amniotic fluid identified presence of intra-amniotic inflammation according to established parameters. NRBC counts were assessed within 1-hour of birth. Early-onset neonatal sepsis (EONS) was established based on hematological and microbiological indices. IL-6, EPO and cortisol levels were measured by immunoassays. Fetal acid-base status was determined within 10 minutes of delivery. Parametric or nonparametric statistics was employed.

Results—Fetuses with EONS (n=19) were delivered at earlier gestational ages (mean±SD: 27.1±2.8 weeks, $P=0.001$) and more often by mothers with intra-amniotic inflammation ($P=0.022$) and histological chorioamnionitis ($P<0.001$). Neonates with EONS had higher absolute NRBCs

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Condensation: In inflammation-associated preterm birth and in the absence of hypoxia, elevated neonatal nucleated red blood cell counts may be a direct response to inflammatory mediators.

Authors' Contributions: Antonette Dulay is the principal investigator and responsible author. Antonette Dulay, Irina Buhimschi and Catalin Buhimschi designed the study, generated, interpreted the data and wrote the initial draft of the manuscript. Guomao Zhao participated with processing of biological samples, performed immunoassays, revised critically the manuscript and approved the final version. Vineet Bhandari participated with aspects of data analysis and interpretation, revised critically the manuscript and approved the final version. Guoyang Luo; Sonya Abdel-Razeq; Michael Cackovic, Victor Rosenberg, Christian Pettker Stephen Thung and Mert Ozan Bahtiyar participated with patient enrollment, acquisition of demographic and clinical data, critical revision of the manuscript and approved the final version.

counts ($P=0.011$). NRBC counts were directly correlated with cord blood IL-6 levels ($P<0.001$) but not with EPO, cortisol or parameters of acid-base status levels regardless of EONS status. These relationships remained following correction for gestational age, diabetes, intrauterine growth restriction (IUGR) and steroid exposure.

Conclusion—In the setting of inflammation-associated preterm birth and in the absence of hypoxia, elevations in NRBCs in the early neonatal period may be a direct response to exposure to inflammatory mediators *in utero*.

Keywords

infection; inflammation; NRBC preterm birth; chorioamnionitis

Introduction

Nucleated red blood cells (NRBC) are immature erythrocytes whose production is thought to be driven primarily by the interplay of hypoxia and erythropoietin (EPO) synthesis [1,2]. EPO is a glycoprotein hormone synthesized by the kidneys, liver, spleen, lung, and bone marrow [3,4]. In human fetuses the liver is the main site of EPO synthesis, with the kidney contributing to production closer to term [3]. Additional sites include the placenta, endothelium, and neuronal cells [3]. However, prior studies suggest that EPO increases erythroid production and releases immature forms of erythrocytes into the peripheral circulation in response to hypoxia [1,5].

The current line of thought is that increased NRBC production in the immediate neonatal state primarily reflects hypoxic injury. [6] Much of the existing literature focuses on hypoxia's contribution to elevated EPO levels, and thus, NRBC counts. Several studies have shown that in cases of classic chronic intrauterine fetal stress, such as preeclampsia and intrauterine growth restriction (IUGR), fetal serum and amniotic fluid EPO levels are elevated [7,8,9,10,11,12]. Such a relationship has led to the prevailing idea that the NRBC count *in utero*, as in the adult state, is mainly driven by hypoxia-triggered EPO release.

However, the mechanisms driving NRBC production may not be as straightforward as previously thought. [13] We have provided evidence in a rat model of IUGR induced by concurrent chronic hypoxia and nitric oxide synthase inhibition, hypoxia *per se* did not play a significant role in fetal erythroblastosis. [14] This made us conclude that an elevation of NRBCs in the fetal circulation does not necessarily serve as a unique marker of chronic hypoxia but may be an epiphenomenon related to inhibition of nitric oxide, oxidative stress or other pathological states such as inflammation. [14]

Sepsis is generally viewed as a disease aggravated by an inappropriate immune response. [15] Previous studies showed that the inflammatory response, characterized by cytokine release, is accompanied by an increased NRBC production. [16] Specifically, it was demonstrated that interleukin-6 (IL-6) is involved. [16] That such responses may occur in pregnancies complicated by intra-amniotic inflammation is supported by the evidence that fetuses delivered for non-reassuring fetal status had normal EPO levels, but elevated IL-6 concentrations. [17] This implies that the fetal inflammatory response and fetal stress may

have distinct roles in NRBC production and/or release in peripheral circulation. Additionally, in pregnancies complicated by histological chorioamnionitis there is suggestion that the inflammatory host response may be the driving force behind fetal NRBC elevation, independent of EPO. [18]

Given that the literature supports roles for hypoxia, EPO, *in utero* stress, and inflammation in NRBC elevation, our objective was to explore simultaneously each of these pathways to isolate the driving forces responsible for the increased NRBC counts in the immediate neonatal period in the context of inflammation-induced preterm birth. We hypothesize that in pregnancies complicated by intrauterine inflammation, the fetal inflammatory response provides the primary impetus behind NRBC elevations with little contribution from hypoxia and EPO.

Methods

Study population

We conducted a prospective study in 68 consecutive, preterm, singleton newborns with no anomalies born to mothers who presented at Yale New Haven Hospital with clinical symptoms of preterm labor or preterm premature rupture of the membranes (PPROM). The study period ranged from May 2004 to July 2007. By study design, neonates were included only if a clinically indicated ultrasound-guided amniocentesis was performed antenatally. The decision to recommend amniocentesis or delivery of the fetus was made by the primary physician, independent of our research protocol. Eligible women met the following inclusion criteria: singleton fetus at <34 weeks gestational age. Exclusion criteria included: anhydramnios, preeclampsia, human immunodeficiency virus or hepatitis infections and presence of fetal heart rate abnormalities at enrolment (bradycardia, prolonged variable decelerations) requiring immediate delivery. The Yale University Human Investigation Research Board approved the study protocol. Written informed consent was obtained from all participants preceding the amniocentesis.

Gestational age was established based on last menstrual period and/or an ultrasonographic examination prior to 20 weeks. Preterm labor was defined as regular uterine contractions associated with advanced cervical dilatation (>3 cm) or effacement at <37 weeks of gestation (19). Rupture of the membranes was confirmed either by direct visualization of amniotic fluid “pooling” on speculum examination, positive “nitrazine” and “ferning” tests, or by a positive amnio-dye test. Clinical chorioamnionitis was diagnosed in the settings of maternal fever (>37.8°C) and at least 1 of the following: uterine tenderness, foul smelling amniotic fluid or visualization of pus at the time of the speculum exam, maternal (>100 beats/min) or fetal tachycardia (>160 beats/min). Following amniocentesis, each woman was followed prospectively up to the point of delivery. Induction of labor or surgical delivery was performed for such clinical indications as a prolapsed umbilical cord, a gestational age <34 weeks in the context of PPRM, clinical chorio-amnionitis and/or amniotic fluid laboratory results (glucose, lactate dehydrogenase (LDH) activity, white blood cell count (WBC), gram stain, microbial cultures) traditionally considered as consistent with intra-amniotic inflammation/infection. [20]

Amniotic fluid microbial cultures were performed for facultative aerobic and anaerobic bacteria, *Ureaplasma* and *Mycoplasma* species. The clinical laboratory performed the glucose and LDH measurements, Gram staining and the white blood cell (WBC) and red blood cell (RBC) counts. The clinical laboratory results were available to the primary care providers for clinical management. After fulfilment of the clinical requirements the remaining amniotic fluid was centrifuged at 3000g, 4°C for 10 min., aliquoted, and stored at -80°C for research purposes.

Diagnosis of intra-amniotic inflammation

We used mass spectrometry SELDI-TOF (surface enhanced laser desorption ionization-time of flight) to confirm or exclude a diagnosis of intra-amniotic inflammation as previously reported. [21] Briefly, the amniotic fluid proteomic fingerprint, the Mass Restricted (MR) score, was generated immediately following the retrieval of amniotic fluid. The MR score provides qualitative information regarding the presence or absence of inflammation. The MR score ranges from 0 to 4, depending upon the presence or absence of each of four protein biomarkers. A value of 1 is assigned if a particular peak is present and 0 if absent. A score of 3 or 4 indicates the presence of “severe” intra-amniotic inflammation. All SELDI-TOF assays were scored “blindly” by investigators unaware of either clinical presentation or outcome.

Determination of the acid-base status at birth

Cord blood samples (umbilical artery and vein) were collected in pre-heparinized 1 cc. syringes, capped and transported to the laboratory. The acid-base status was determined within 10 min. of delivery with the ABL 800 FLEX blood gas analyzer (Radiometer Medical A/S, Denmark). For research purposes, umbilical cord blood specimens were retrieved in plain and blue-top tubes, spun at 3000g, 4°C for 10 min., aliquoted and stored at -80°C, until analyzed for IL-6, EPO and cortisol using sensitive ELISA assays.

Nucleated red blood cell count and units of reporting

Within the first hour of life, neonatal whole blood was collected in a purple topped EDTA tube for a complete cell blood count (CBC) and NRBC count. We used manual microscopy to count the NRBCs for which a drop of blood was smeared over a glass slide and stained by the Wright method. [22] The hematology laboratory results were reported as NRBC per 100 WBC. [23,24] Traditionally and as prior studies have shown, NRBC expression per 100 WBC can introduce bias especially in inflammatory states when changes in WBC counts are expected. [25] Therefore for the purpose of this analysis the reported NRBC values were converted to an absolute NRBC count per volume of blood and analysed as NRBC/mm³.

Immunoassays procedures

Interleukin-6—ELISA for human IL-6 (Pierce-Endogen, Rockford, IL) was performed in duplicate according to manufacturers' instructions in both AF and umbilical cord blood. The minimal detectable concentration was 1-pg/mL and the inter- and intra-assay coefficients of variation <10%.

Erythropoietin—ELISA for EPO (R&D Systems, Minneapolis, MN) was performed in duplicate according to the manufacturer's instructions. The minimal detectable concentration for EPO was 0.6 mIU/mL. The inter- and intra-assay coefficients of variation were <10% for EPO.

Cortisol—ELISA for cortisol (R&D Systems, Minneapolis, MN) was performed in duplicate according to the manufacturer's instructions. The minimal detectable concentration for cortisol was 0.071 ng/mL. The inter- and intra-assay coefficients of variation were <7%.

Histological evaluation of the placenta for acute inflammation

In our clinical setting pathological examination of the placenta is a routine part of the evaluation of a pregnancy complicated by preterm labor or PPRM. Placental tissues were available for all 68 neonates. Sections were read by a perinatal pathologist unaware of the results of the proteomic profiling or the results of the amniotic fluid or umbilical cord analyses. Three histological stages of chorioamnionitis (stage I: intervillitis, stage II: chorionic inflammation, and stage III: full thickness inflammation of both chorion and amnion) were complemented by the histological grading system devised by Salafia et. al. which includes four grades of inflammation of the amnion, chorion-decidua, and umbilical cord. [26, 27]

Evaluation of early-onset neonatal sepsis

Neonatal hematological indices and sepsis categorization were assessed for all neonates as previously described, from blood specimens and cultures obtained immediately following delivery, by an investigator unaware of the results of the proteomic profiling. [28,29] All 68 neonates underwent clinical and laboratory evaluations of EONS, defined as the presence of confirmed or suspected sepsis within the first hour of life. Clinical indicators of EONS included: lethargy, apnea, respiratory distress, hypoperfusion and shock. Confirmed sepsis was established when either the blood and/or cerebrospinal fluid microbiological culture was positive. Suspected sepsis was diagnosed in the presence of clinical suspicion of sepsis with support from laboratory results. Laboratory criteria were based on modification of the criteria of Rodwell et al. when two or more of the following were observed: (absolute neutrophil count (ANC) <7,500/mL or >14,500/mL, absolute band count (ABC) >1,500/mL, immature/total neutrophil ratio (I:T) ratio>0.16, platelet count<150,000 cells/mm³ or abnormal cerebrospinal fluid results. [28] All neonates with suspected sepsis received antibiotic therapy. EONS was dichotomized into present (when sepsis was either confirmed or suspected) or absent and coefficients of associations with other binary variables were calculated.

Statistical analysis

The Kolmogorov-Smirnov test was used for data normality testing. Statistical analyses were performed with Sigma Stat, version 2.03 (SPSS Inc., Chicago, IL) and MedCalc (Broekstraat, Belgium) statistical software. Comparisons between groups were performed using Student t-tests or Mann-Whitney rank sum tests. Proportions were compared with Fisher's exact or Chi square tests. Multiple stepwise linear regression analyses were used to adjust *p* values for potential influences of gestational age or other parameters. Variables

were entered into the model if $P < 0.05$ and removed if $P > 0.1$. The IL-6, cortisol, EPO concentrations are presented as an arithmetic median. Statistical analysis was completed following logarithmic transformation of the cytokine levels before correlation analyses using Pearson product moment correlation coefficient. For sample size calculation we assumed that an increased NRBC ($> 1000 \text{ NRBC/mm}^3$) will be able to correctly predict EONS in 80% of cases. [13] We estimated a minimum required sample size of 18 neonates with EONS would be necessary to detect 25% differences between groups, given 80% power and a confidence coefficient of 95%. A P value of < 0.05 was used to indicate significance.

Results

Study population, intra-amniotic inflammation and EONS

Demographic, clinical and pregnancy outcome characteristics are presented in Table 1. Women who delivered fetuses with EONS were of lower gestational ages at amniocentesis. Neonates with EONS were delivered earlier, had lower birth-weights and lower Apgar scores. Placental histological examination of the neonates diagnosed with EONS more frequently showed presence of histological chorioamnionitis. In Table 2 we present the results of the amniotic fluid analysis. Amniocentesis results indicated that mothers whose infants had EONS, had lower glucose levels, higher LDH activity, higher WBC counts and more often a positive Gram stain and culture result. Amniotic fluid of EONS neonates had significantly higher IL-6 concentrations and higher MR scores indicative of intra-amniotic inflammation by proteomic analysis. All of these differences remained significant following correction for gestational age at amniocentesis or delivery.

Umbilical cord blood gas analysis

Table 3 summarizes the findings from umbilical cord blood gas analysis. Neonates affected by EONS had higher umbilical artery pH and lower $p\text{CO}_2$. Infants with EONS also had higher umbilical vein $p\text{O}_2$ and lower $p\text{CO}_2$ with no significant changes in pH. However, each of these associations disappeared after correcting for gestational age at delivery ($P > 0.05$).

Neonatal hematological indices, absolute NRBC count and reticulocyte count

Table 4 displays our findings regarding the hematological profile of the 68 neonates at birth. Of these ANC, ABC and I:T ratio are components of EONS definition. [28] Neonates with EONS were anemic, lymphopenic, had higher WBC counts, I:T ratios, and higher percentages of circulating immature neutrophils (bands). After correcting for gestational age, the associations found between EONS and WBC count and ANC were not significant ($P > 0.05$). In contrast, the associations with ABC, hematocrit, hemoglobin, bandemia, lymphocytes, and I/T ratio continued to remain significant.

The absolute NRBC count of neonates with EONS was significantly higher compared to those neonates who were not diagnosed as such ($P = 0.011$). Moreover, this finding remained significant after correcting for gestational age at birth. Additionally, babies affected by EONS had an elevated reticulocyte count that remained significant following correction for hematocrit and gestational age at delivery in multivariate regression ($P = 0.009$).

Inflammatory and stress status of the fetus at birth

Determination of the fetal inflammatory status as reflected by cord blood IL-6 showed that neonates with EONS had much higher IL-6 levels at birth compared with those without EONS. Conversely, both groups of neonates had similar cortisol levels. These relationships remained valid after correction for gestational age at delivery.

Umbilical cord blood erythropoietin levels

At birth neonates with EONS had umbilical cord EPO levels no different than neonates without evidence of EONS ($P = 0.436$). This relationship persisted after correction for gestational age and results of cord gas analysis at the time of delivery.

Relationships between cord blood IL-6, cortisol and EPO levels, and absolute NRBC counts

We found a significant correlation between NRBC counts and cord IL-6 levels ($r=0.442$, $P < 0.001$) (Figure 1A) but not between NRBCs and either EPO ($r=0.080$, $P = 0.534$) (Figure 1B) or cortisol concentrations ($r=0.052$, $P = 0.677$). In a multivariate linear regression analysis with absolute NRBC count as dependent variable and cord blood IL-6, cortisol, EPO gestational age at delivery, maternal age, diabetic status, fetal IUGR, antenatal corticosteroid exposure, histological chorio-amnionitis and mode of delivery as independent variables, only the cord IL-6 levels were retained in the model at a $P < 0.001$ while all the other variables were excluded based on a $P > 0.100$.

Discussion

We present evidence that the fetal hematological response to inflammation is complex. To test our hypothesis that fetal inflammation is associated with an increased NRBC count in pregnancies complicated by preterm birth, we evaluated the fetal hematopoietic system by examining fetal cord blood at the time of birth and related our results to hematological indices and sepsis categorization of the neonate within the first hour of life. The elevation of NRBC count directly correlated with cord blood IL-6, but not acid-base status, EPO or cortisol levels. We have demonstrated, and, to our knowledge for the first time, that in the context of inflammation-induced preterm birth and in the absence of hypoxia or stress, fetal erythroblastosis and/or the release of NRBCs in peripheral circulation may be an adaptive response to an inflamed intrauterine environment. After correcting for gestational age, maternal diabetes, IUGR, mode of delivery, antenatal corticosteroid and antibiotic exposure, the above relationships remained significant. We should point out that in our population we had only 1 case of IUGR, preeclampsia cases were excluded by study design, and no cases of acidosis ($\text{pH} < 7.0$) were encountered, as would be expected in an acute tertiary care obstetrical setting and in keeping current standard of care.

Accurate and timely diagnosis of the newborn with suspected sepsis is essential to prevent early death and long-term neuro-developmental complications. [30] Evidence in the last two decades has demonstrated that intra-partum and peripartum antibiotic prophylaxis is effective in preventing group B streptococcus sepsis. This provides explanation and support for the use of antibiotics in populations at risk. [31] In the era of widespread antepartum and

intra-partum chemoprophylaxis, this approach has resulted in the development of multidrug resistance, changes in pathogens causing EONS, and increased difficulty in confirmation of neonatal sepsis based on positive blood cultures. [32] In an effort to avoid unnecessary antibiotic administration, attention has turned toward new diagnostic approaches such as the description of biomarkers and hematological indices designed to identify neonates at risk for sepsis and poor outcome. [21,28,30] The importance of this concept is predicated by studies which demonstrate that general hematological indices and NRBCs are good predictors of short term neonatal outcome, independent of gestational age or birth weight. [4,33,34] This suggests that at birth, description of hematological indices and serologic markers even in the absence of positive cultures carries not only diagnostic and prognostic value, but also provides insight into the pathophysiology of fetal adaptation to a microbial inflammatory attack.

Much of the current literature focuses on the contribution of hypoxia to the fetal production of NRBCs at term. [35] Traditionally it has been thought that EPO, in response to hypoxia, is the main driving force behind NRBC production. [36, 37] It has also been thought that the duration and severity of fetal hypoxia may be predicted by the number of increased NRBCs in the neonates delivered both preterm and at term. [12,33,38] Recent data has questioned whether hypoxia is the sole mechanism responsible for an increase in the fetal NRBC. [14, 39] This parameter's etiology may be multifactorial with many pathophysiological pathways involved such as oxidative stress, hypoxia, and inflammation.

Although previous studies have proposed that inflammation may play a role in elevating the fetal NRBC count in the context of PPROM or histological chorioamnionitis, the mechanism through which this occurred remained unclear. Earlier studies, however, were unable to assess simultaneously amniotic fluid and/or cord blood cytokine levels. [17,18,40]. The results of the current study support the concept that cytokines have an important role in stimulating fetal NRBC production, independent of hypoxia. This paradigm is strengthened by several other lines of evidence. For example, when recombinant IL-6 was given to adult animals, a selective erythroid hyperplasia of the marrow's late normoblasts was observed in the first 12 hours following cytokine administration. [41] This time frame favors a direct effect of IL-6 *in vivo* rather than via induction of EPO. Similarly, increased NRBC counts have been reported in gravely ill critical care patients in the context of increased cytokine (IL-3, IL-6, IL-12) levels. [16] Collectively, these studies speak to a possible direct role of inflammatory cytokines in stimulating erythropoiesis.

At this time, we do not wish to propose that an elevated absolute NRBC in preterm neonates is a specific marker of EONS, but rather, to suggest that other factors independent of hypoxia and acidemia, such as *in-utero* exposure to an inflammatory environment, impacts on neonatal NRBCs. By study design, we evaluated the NRBCs at the time birth, and thus the results of our study are mostly reflective for factors impacting on the fetus while *in utero*. After birth, NRBC counts are also expected to be affected by postnatal interventions (i.e. oxygenation, antibiotics, surfactant, corticosteroids, anti-inflammatories etc.) of the preterm neonate. Longitudinal studies remain to be completed in order to address the impact of such interventions on NRBC counts.

In summary, we propose that inflammation has an important role in the elevation of NRBCs in newborns delivered in the context of inflammation-induced preterm birth, independent of EPO or hypoxia. It is still unknown whether it is the pathological stimulation or injury of the erythroid production sites *in utero* by the inflammatory response or a stimulation of the release of immature forms of erythrocytes into the peripheral circulation that leads to the elevation in NRBC in the peripheral blood. However, our data suggests that IL-6 may be a trigger for NRBC release given that neonates with intra-amniotic inflammation also had a lower red cell mass. The long-term consequences for such a finding remain to be identified.

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Figure 1. Relationships between cord blood IL-6, EPO and NRBC count

A. Correlation analysis of cord blood interleukin-6 (IL-6) and absolute nucleated red blood cell (NRBC) count in logarithmic format. **B.** Correlation analysis of cord blood erythropoietin (EPO) and absolute NRBC count in logarithmic format. The regression line (solid black) and 95% confidence (solid red lines) and prediction (dotted red lines) intervals are also shown (n=68).

Table 1
Demographic and clinical characteristics of women and their newborns

Variable	All n=68	EONS		P value
		No n=49	Yes n=19	
Maternal characteristics at enrollment and hospital course				
Age, years †	28.3 ± 7.0	28.6 ± 6.8	27.5 ± 7.6	0.564
Gravidity ‡	3 [2-5]	3 [1-5]	3 [2-5]	0.420
Parity ‡	1 [0-2]	1 [0-2]	1 [0-2]	0.516
Race §				0.453
Caucasian	28 (41)	23 (34)	5 (7)	
African-American	22 (32)	14 (21)	8 (12)	
Hispanic	14 (21)	9 (13)	5 (7)	
Other	4 (6)	3 (4)	1 (1)	
Gestational age, weeks †	28.2 ± 3.1	28.8 ± 3.1	26.7 ± 2.4	0.009
Ruptured membranes §	37 (54)	30 (61)	7 (37)	0.124
Presence of uterine contractions §	31 (46)	21 (43)	10 (53)	0.649
Clinical chorioamnionitis	7 (10)	5 (10)	2 (11)	1.000
History of preterm birth §	24 (35)	17 (35)	7 (37)	0.907
Steroid exposure during pregnancy §	67 (98)	48 (98)	19 (100)	1.000
Prenatal antibiotic treatment §	62 (91)	45 (92)	17 (89)	1.000
Diabetes §	2 (3)	1 (2)	1 (5)	1.000
IUGR §	1 (1)	1 (1)	0 (0)	1.000
Outcome characteristics				
Amniocentesis-to-delivery, hours ‡	11.7 [4.1-87.8]	23.0 [4.5-104.7]	5.2 [4.1-18.2]	0.094
Gestational age at delivery, weeks †	28.9 ± 3.0	29.6 ± 2.7	27.1 ± 2.8	0.001
Birthweight, grams †	1346 ± 476	1451 ± 442	1077 ± 464	0.003
Cesarean delivery §	35 (52)	27 (55)	8 (42)	0.489
Apgar score at 1 minute ‡	7 [4-8]	7 [5-8]	5 [2-6]	0.002
Apgar score at 5 minutes ‡	8 [7-9]	9 [8-9]	7 [6-8]	< 0.001
Male sex §	37 (54)	29 (59)	8 (42)	0.319
Histological chorioamnionitis §	35 (51)	18 (37)	17 (89)	< 0.001

[†]Data presented as mean ± standard deviation and analyzed by Student t-tests.

[‡]Data presented as median [interquartile range] and analyzed by Mann Whitney tests.

§Data presented as n (%) and analyzed by Chi square tests.

¶Data presented as n (%) and analyzed by Fisher's exact tests.

Abbreviation: IUGR: intrauterine growth restriction

Table 2
Amniotic fluid analysis

Variable	All n=68	EONS		P value
		No n=49	Yes n=19	
Glucose, mg/dL ‡	15 [3-28]	19 [11-33]	3 [2-12]	<0.001
LDH activity, U/L ‡	322 [169-725]	267 [150-464]	886 [283-2,248]	0.005
WBC count, cells/mm ³ ‡	45 [7-983]	32 [4-223]	1020 [37-3,410]	0.002
RBC count, cells/mm ³ ‡	144 [7-1,160]	261 [10-1,620]	96 [4-762]	0.266
Positive Gram stain §	20 (29)	8 (16)	12 (63)	<0.001
Positive cultures §	26 (38)	11 (22)	15 (79)	<0.001
IL-6, ng/mL ‡	7 (10)	6.1 [0.5-12.1]	33.4 [4.8-82.7]	0.005
MR score ‡	3 [2-4]	2 [1-4]	4 [2-4]	0.022

‡ Data presented as median [interquartile range] and analyzed by Mann Whitney tests.

§ Data presented as n (%) and analyzed by Chi square tests. MR: mass restricted score. The MR score ranges from 0 to 4. A score of 3-4 indicates the presence of "severe" inflammation, while a score of 0-2 excludes it.

Abbreviations: LDH lactate dehydrogenase; WBC: white blood cell count; RBC red blood cell; IL-6: interleukin-6; MR: Mass Restricted

Table 3
Umbilical cord blood gas analysis

Variable	All n=68	EONS		P value
		No n=49	Yes n=19	
Umbilical artery				
pH $\ddagger$	7.32 [7.29-7.34]	7.32 [7.27-7.33]	7.33 [7.32-7.36]	0.029
pO2, mmols/L $\ddagger$	24.5 [19.2-33.5]	23.6 [18.4-28.0]	31.2 [22.0-38.0]	0.050
O2 sat, % $\ddagger$	45.7 [32.2-30.7]	42.3 [28.2-58.4]	61.6 [38.7-78.1]	0.050
pCO2, mmols/L $\ddagger$	42.4 [39.3-48.7]	45.3 [40.0-49.6]	40.4 [35.6-43.5]	0.046
HCO3, mmols/L $\ddagger$	21.3 [19.9-22.5]	21.3 [20.2-22.6]	20.3 [19.4-21.8]	0.489
base deficit, mmols/L $\ddagger$	4.3 [2.4-5.4]	4.3 [2.4-5.4]	4.2 [2.5-5.5]	0.558
Umbilical vein				
pH $\ddagger$	7.36 [7.33-7.39]	7.35 [7.33-7.39]	7.38 [7.34-7.40]	0.117
pO2, mmols/L $\ddagger$	31.3 [26.5-39.8]	30.8 [26.1-37.7]	38.7 [30.3-49.6]	0.041
O2 sat, % $\ddagger$	65.4 [51.2-80.3]	62.1 [50.5-77.7]	71.5 [61.7-91.9]	0.104
pCO2, mmols/L $\ddagger$	37.6 [35.6-42.5]	38.8 [36.2-43.2]	34.5 [29.2-38.6]	0.028
HCO3, mmols/L $\ddagger$	21.2 [19.7-22.2]	21.3 [19.8-22.2]	20.2 [18.6-22.7]]	0.422
base deficit, mmols/L $\ddagger$	3.6 [2.2-4.8]	3.5 [2.2-4.7]	3.9 [2.1-5.4]	0.682

[‡]Data presented as median [interquartile range] and analyzed by Mann Whitney tests.

Table 4
Results of neonatal haematological indices and cord blood cytokines

Variable	All n=68	EONS		P value
		No n=49	Yes n=19	
<i>Hematological indices</i>				
Hematocrit % †	46.6 ± 7.0	48.3 ± 6.6	42.1 ± 6.0	<0.001
Hemoglobin, g/dL †	15.1 ± 2.2	15.6 ± 2.0	13.6 ± 2.1	<0.001
WBC count, cells × 1000/mm ³ ‡	10 [7-14]	9 [7-12]	13 [10-20]	0.019
Platelets, cells × 1000/mm ³ ‡	255 [227-314]	259 [229-307]	239 [206-335]	0.662
Segmented, % †	33 ± 14	35 ± 14	30 ± 14	0.264
Bands, % ‡	5 [2-13]	3 [1-6]	20 [14-26]	<0.001
Lymphocytes, % ‡	42 ± 16	45 ± 15	32 ± 13	0.002
ANC, cells/mm ³ ‡	3359 [2094-4,886]	3,330 [2,097-4,460]	3,875 [1974-6027]	0.436
ABC, cells/mm ³ ‡	42.4 [39.3-48.7]	45.3 [40.0-49.6]	40.4 [35.6-43.5]	0.046
I:T ratio, % ‡	5 [2-13]	3 [1-6]	20 [14-26]	<0.001
Positive blood cultures §	5 (7)	0 (0)	5 (26)	0.001
<i>Nucleated red blood cells</i>				
Absolute NRBCs, cell/mm ³ ‡	1,526 [682-3,084]	1,330 [665-2,630]	3,020 [1,388-4,558]	0.011
Reticulocyte count, cells/100 RBCs ‡	7.6 ±2.2	7.1 ±2.0	9.7 ±2.0	0.002
<i>Umbilical cord blood immunoassay results</i>				
IL-6, pg/mL ‡	13.2 [6.1-93.5]	7.4 [5.8-33.1]	90.2 [17.9-407.5]	<0.001
Cortisol, ng/mL ‡	72.5 ± 35.2	73.2 ± 34.8	70.9 ± 37.1	0.812
EPO, mUIg/mL ‡	7.0 [4.0-17.8]	6.6 [4.0-15.2]	11.3 [3.7-30.8]	0.436

[†] Data presented as mean ± standard deviation and analyzed by Student t-tests.

[‡] Data presented as median [interquartile range] and analyzed by Mann Whitney tests.

Abbreviations: WBC white blood cell; ANC absolute neutrophils count; ABC absolute band count; I:T immature-to-total neutrophil ratio; NRBC nucleated red blood cell; IL-6 interleukin-6; EPO erythropoietin