KORDEK, Agnieszka, BŁAŻEJCZAK, Anna and KORDEK, Justyna. Usefulness of estimation of cord blood lipopolysaccharide binding protein and neutrophilic elastase concentration versus C-reactive protein concentration in the prediction of early-onset neonatal infections. Journal of Education, Health and Sport. 2025;80:57632. eISSN 2391-8306.
https://doi.org/10.12775/JEHS.2025.80.57632
https://apcz.umk.pl/JEHS/article/view/57632

The journal has had 40 points in Minister of Science and Higher Education of Poland parametric evaluation. Annex to the announcement of the Minister of Education and Science of 05.01.2024 No. 32318. Has a Journal's Unique Identifier: 201159. Scientific disciplines assigned: Physical culture sciences (Field of medical and health sciences); Health Sciences (Field of medical and health sciences).
Punkty Ministerialne 40 punktów. Załącznik do komunikatu Ministra Nauki i Szkolnictwa Wyższego z dnia 05.01.2024 Lp. 32318. Posiada Unikatowy Identyfikator Czasopisma: 201159. Przypisane dyscypliny naukowe: Nauki o kulturze fizycznej (Dziedzina nauk medycznych i nauk o zdrowiu); Nauki o zdrowiu (Dziedzina nauk medycznych i nauk o zdrowiu).
The authors declare that there is no conflict of interests regarding the publication of this paper.
Received: 14.01.2025. Revised: 21.03.2025. Accepted: 04.04.2025. Published: 04.04.2025.

Usefulness of estimation of cord blood lipopolysaccharide binding protein and neutrophilic elastase concentration versus C-reactive protein concentration in the prediction of early-onset neonatal infections

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Abstract

Introduction. Congenital infection in a newborn is an infection caused by pathogens passed on to the child by the mother intrauterine or perinatally. Sepsis is the most common form of early-onset infection in newborns. The diagnosis in the newborn is difficult due to the absence of characteristic clinical signs as well as specific and infection-specific laboratory deviations.

Objective. The study was conducted to estimate the predictive value of cord blood plasma concentration of lipopolysaccharide-binding protein, and neutrophil elastase in the prediction of early onset sepsis in preterm and full-term neonates. Correlation with C-reactive protein was assessed.

Materials and methods. Patients enrolled in the study (n=153) were divided in 4 groups: B-I- 39 infected preterm; B-II- 24 infected full-term; K-I- 45 non-infected preterm and K-II- 45 full-term neonates without signs of infection. The research material was umbilical cord taken immediately after delivery in the presence of risk factors for infection and without risk factors for obtaining a control group. The concentration of lipopolysaccharide binding protein, activity of neutrophilic elastase and CRP concentration were determined in the collected samples.
Results. The cord blood plasma LBP and neutrophil elastase concentration was higher in infected preterm and term neonates. Cord blood LBP is a highly specific and sensitive biomarker of sepsis immediately after birth. High levels of LBP and neutrophil elastase in cord blood correlate with CRP.
Conclusions. Measuring the concentration of LBP and neutrophil elastase in cord blood plasma may help in the detection of early onset sepsis in newborns with prenatal risk factors.

Keywords: C-reactive protein, early-onset bacterial infection, lipopolysaccharide-binding protein, neutrophil elastase, newborn,

Introduction

Congenital infection in a newborn is an infection caused by pathogens passed on to the child by the mother intrauterine or perinatally [1]. According to various authors, the frequency of early-onset neonatal sepsis (EOS) ranges from 1-10 ‰, and in the case of children with very low body weight (410-1500 g) to 15-19‰ [2-4]. Sepsis is the most common form of early-onset infection in newborns.

The diagnosis of congenital infection in the newborn is difficult due to the absence of characteristic clinical signs as well as specific and infection-specific laboratory deviations. In immature, hypoxic newborns, disease progression can rapidly lead to death. It happens that sepsis in a newborn begins and ends with death in a clinical picture of septic shock. The same symptoms of organ failure may accompany other, non-infectious disease states typical of the neonatal period: respiratory distress syndrome, low cardiac output syndrome, metabolic disease, ion disorders, hypoglycemia, dehydration, perinatal trauma or intracranial bleeding [5].
It has been shown that in the early postnatal period, the values of some biochemical inflammatory parameters show some differences. This applies mainly to procalcitonin, but also to interleukin-6, CRP, and WBC, which may cause difficulties in the interpretation of test results [6,7]. Blood cultures recognized as the golden standard in the diagnosis of septic infections are not helpful in making a quick decision to start antibiotic therapy because of the

time needed to obtain a positive result. Negative blood cultures can be obtained in 38-70% of newborns diagnosed with suspected sepsis [8,9]. Obtaining a positive blood culture is often unrealistic in the age of widely used perinatal antibiotics. Moreover, bacteremia can be transient, intermittent, too little blood has been drawn, or has been improperly processed [10].

Advances in molecular microbiology allow the use of the PCR (polymerase chain reaction) method - to detect bacterial genetic material, but it is an expensive method, limited by the risk of contamination, the inability to assess bacterial resistance, i.e. obtaining an antibiogram, and is not widely available [11,12].

The inability to unequivocally determine the state of infection at its early stage with a real risk of sepsis often leads to too hasty administration of the antibiotic. Therefore, the search for infection markers characterized by rapid growth dynamics in the initial stage of infection, a sufficiently long half-life, high specificity and sensitivity, as well as ease of use in everyday clinical practice and reasonable costs, still attracts the attention of neonatologists.
The most popular biochemical parameter routinely used in clinical practice is C-reactive protein, one of many known acute-phase proteins.

Protein binding lipopolysaccharide and neutrophilic elastase are biomarkers whose usefulness in the diagnosis of infections in various clinical situations is under investigation.
Lipopolysaccharide (LPS)-binding protein (LBP) was initially discovered as an acute-phase reactant binding with the LPS of Gram-negative bacteria walls to form LPS-LBP complexes and was later found to be elevated in Gram-positive bacteremia [13]. Neutrophilic elastase is an enzyme of the granularity of azurophilic multinucleated granulocytes. It belongs to the proteases. Neutrophilic elastase is involved in the inflammatory response and, together with free radicals, causes local tissue damage. The increase in the concentration of the elastase-α-1-proteinase inhibitor complex occurs 4-6 hours after the action of the infectious agent and reaches its maximum after 18-24 hours. The increase in the concentration of elastase in the blood is proportional to the degree of neutrophil activity and the intensity of inflammation [14]. The elastase-α-1-antitrypsin complex is very stable and can be easily identified in an enzyme test.

Objectives. The study was conducted to estimate the predictive value of cord blood plasma concentration of lipopolysaccharide-binding protein, and neutrophil elastase in the prediction of early onset sepsis in preterm and full-term neonates. Correlation with C-reactive protein was assessed.

Material and methods

The prospective research was carried out in an academic center with reference level III after obtaining the consent of the Bioethics Committee (BN-001/15/05) and informed written consent of the parents (according to the Helsinki Declaration). 153 newborns were enrolled in the study. Out of this group 63 were diagnosed with early-onset infection (EOS), 39 of them were premature (B-I) and 24 were born at term (B-II). The control group consisted of uninfected newborns, premature (K-I, n = 45) and full-term (K-II, n = 45) infants, respectively. The inclusion criteria for the study group was the diagnosis of EOS based on clinical symptoms and laboratory test results.

Early-onset sepsis occurred with a frequency of 4.7% in the group of premature newborns (833 born during the study) and 1.4% in the group of full-term newborns (1,666 born). The inclusion criteria for the study groups were: the presence of prenatal risk factors for intrauterine infection, the presence of abnormal clinical symptoms in the first 72 hours of a child's life, and deviations in the results of laboratory, imaging and microbiological tests.
Chorioamnionitis was suspected in the presence of the following clinical symptoms: maternal fever> 38 ° C accompanied by uterine pressure soreness, maternal or fetal tachycardia, and foul purulent amniotic fluid [1]. Additionally, premature departure of amniotic fluid beyond 12 hours was taken into account, and in the case of the mother: leukocytosis> $12.0x10^9$ / L, CRP> 10 mg / L, after excluding foci of infection not related to the fetus [1,15].

Designation of infection status.

All newborns were examined by a neonatologist as frequently as required, at least once a day. Early-onset infection was diagnosed when symptoms appeared in up to 72 hour of life. Clinical sepsis was recognized based on the presence of three or more of the following five categories of clinical signs: 1. skin color (pallor, jaundice, cyanosis); 2. respiratory function (apnea, tachypnea >60/min, grunting, nasal flaring, intercostal or sternal retractions, need for high ventilator settings or oxygen); 3. cardiovascular function (brady/tachycardia, poor peripheral perfusion, hypotension); 4. neurologic findings (hypotonia, irritability, lethargy, seizures); 5. gastrointestinal function (abdominal distension, green or bloody residuals, vomiting; temperature instability) [16]. Positive blood culture was not a necessary requirement for diagnosis of early onset sepsis [17, 18]. Positive cord blood culture was obtained in only 4 cases: 2x *Streptococcus agalactie, Escherichia coli, Enterococcus foecalis*.

Other investigations performed when clinically indicated included chest or abdominal radiographs, urine and spinal fluid microscopy and culture, tracheal aspirate culture and cultures from superficial sites. In addition, standard laboratory biochemical tests (eg. glucose and protein levels) and visual examinations were assessed.

Laboratory tests routinely performed in the management of infection included C-reactive protein levels (CRP values > 5 mg/L in neonate's venous blood were considered abnormal), white blood cell count with differential (WBC > 25 or < 5 G/L were considered abnormal), platelet count (Plt < 100 G/L were considered abnormal), and the immature to total neutrophil ratio (I:T ratio > 0.2 were considered abnormal) [19].

Respiratory failure was diagnosed in newborns that required oxygen supplementation and respiratory support by a non-invasive or invasive method.

The criterion excluding from the study were birth defects and chromosomal aberrations.

The control groups included newborns without risk factors for congenital infection, born via vaginal routes and elective caesarean section due to maternal cardiological or ophthalmological indications.

The research material was umbilical cord blood (5 ml) taken immediately after delivery in the presence of risk factors for infection and without risk factors for obtaining a control group. Qualification to the study and control groups was carried out retrospectively, after the diagnosis of the child was established. Blood was collected in tubes containing an anticoagulant (EDTA-K2 potassium edetate), and then centrifuged for 10 min at 5000 rpm. The obtained plasma was stored at -80ºC until the measurements. The concentration of lipopolysaccharide binding protein, activity of neutrophilic elastase and CRP concentration were determined in the collected samples. In newborns diagnosed for EOS, biochemical, microbiological and radiological tests were performed in accordance with the procedure adopted in the clinic.

Lipopolysaccharide binding protein concentration was quantified by enzyme immunoassay method (ELISA -Hycult Biotechnology). The concentration of human α1-antitrypsin bound neutrophilic elastase was determined by ELISA - Human PMN Elastase ELISA (BioVendor; Brno; Czech Republic). Serum C-reactive protein (CRP) concentration was determined by the quantitative immuno-turbidometric method using the Olympus AU 560 System (Olympus diagnostica, Hamburg, Germany). The value below 5 mg / L was adopted as the norm.

Statistical analysis

The normality of the distribution of continuous variables was examined with the Kolmogorov-Smirnow test. These variables were described by means, standard deviations, medians, quartiles, and minimum and maximum values. The statistical differences between the two groups were checked using the Student's t-test for independent variables or Mann-Whitney's. For multiple groups analysis of variance (ANOVA) or Kruskal-Wallis test was used.

The significance of differences between two examinations of the same patients was checked with the Student's t-test for dependent variables or Wilcoxon. The results were described by the value of the difference between the studies (mean and standard deviation) and the probability. Discontinuous variables are described by number and frequency. The $\chi 2$ Pearson test was used to examine the statistical dependencies between discontinuous variables. Spearman's rank correlation and Pearson's correlation were used to examine the correlation between discontinuous variables: ordinal and nominal (coded variables: 0/1) and continuous variables. The results were described by the correlation coefficient r and probability p.

The distributions of variables between the groups were examined using the Kolmogorov-Smirnow test. The results were described by the maximum difference between the distributors and the probability p. For the variables for which the maximum differences were obtained (in relation to the reference - control group), the ROC (Receiver Operating Characteristic Curves) analysis was used. The value for which the sum of sensitivity and specificity was the highest was assumed as the cutoff point. The results were described by specifying the area under the curve, the probability p, and the coordinates of the ROC curves, i.e. for individual ranges of the value of the continuous variable, the sensitivity and specificity of the pathology relative to the control group were estimated. Statistically significant differences in all tests were those for which the probability $p < 0.05$.

Statistical analyzes were carried out using the statistical program STATA 11, license number 30110532736.

Results

Table 1 shows the demographic and clinical characteristics of participants.

Table 1.

Demographic and clinical characteristics	Group B-I N=39	Group K-I N=45	Group B-II N=24	Group K-II N=45
Birth weight (g); X±SD	1561 ± 589	2156 ± 538	3355 ± 516	3412 ± 503
Gestational age; X±SD	30 ± 3	33 ± 3	39 ± 1	39 ±1
Number of newborns < 30 Hbd; N(%)	16 (41)	7 (16)	-	-
Number of newborns with body weight <1000 g; N (%)	8 (21)	0	-	-
Prenatal steroid therapy; N (%)	12 (31)	5 (11)	-	-
Chorioamnionitis; N (%)	7 (18)	0	1 (4)	0
Prenatal antibiotic therapy; N (%)	32 (82)	23 (51)	3 (13)	5 (11)
PROM; N (%)	25 (64)	27 (60)	8 (33)	21 (47)
The outflow of amniotic fluid (h); X±SD	71 ± 100	74±96.5	14.2 ± 4.6	16.9±16
Cesarean section; N (%)	27 (69)	17 (38)	5 (21)	19 (42)
Emergency caesarean section; N (%)	6 (15)	4 (9)	2 (8)	0
Apgar score in the first 5 min.; X±SD	6 ± 1	9±1	8±2	9±1
Apgar score < 5 in the first 5min; N (%)	2 (5)	0	0	0
Males; N (%)	23 (59)	25 (56)	17 (71)	22 (49)
Respiratory failure; N (%)	29 (74)	8 (18)	9 (38)	0
Death of the newborn; N (%)	2 (5)	0	0	0

Legend: PROM – Premature Rupture of Membranes

The results of the ROC curve analysis are presented in Tables 2 and 3. The ROC analysis showed, a good for LBP (AUC from 0.80 to 0.90) and a weak (AUC from 0.60 to

0.70) for neutrophilic elastase, discriminatory capacity in umbilical cord blood for predicting early-onset infection in full-term and premature newborns.

Table 2. Analysis of selected ROCs for neonates with EOS born prematurely (B-I)

Curve parameters	LBP	Elastase	CRP
Area under the curve (AUC)	**0.841**	0.619	0.685
Standard deviation	0.044	0.049	0.41
Probability - p	**<0.001**	**0.017**	**<0.001**
Confidence interval (95%)	0.755-0.927	0.523-0.715	0.605-0.766

Table 3. Analysis of selected ROCs for newborns with EOS (B-II) born at term

Curve parameters	LBP	Elastase	CRP
Area under the curve (AUC)	**0.886**	0.701	0.776
Standard deviation	0.056	0.056	0.047
Probability - p	**<0.001**	**0.002**	**<0.001**
Confidence interval (95%)	0.776-0.996	0.590-0.811	0.684-0.868

Table 4. Cut-off point, sensitivity, specificity, PPV, NPV for LBP, neutrophilic elastase, cord blood CRP in predicting EOS

Biomarker	Group	Cut-off point	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
LBP	B-I	> 11.97 [µg/ml]	71	97	94	83
	B-II		80	99	95	93

Elastase	B-I	>45.06 [µg/l]	41	73	50	60
	B-II		54	73	22	76
CRP	B-I	>4.4 [mg/l]	40	94	84	66
	B-II		51	94	96	83

Table 4 shows the cut-off values, sensitivity, specificity, PPV and NPV for the studied congenital infection biomarkers. It can be noted that the highest values of sensitivity and specificity were obtained for LBP at the cut-off point > 11.97 µg / ml.

In full-term newborns, a significant positive correlation was found between both LBP and neutrophil elastase and CRP levels in umbilical cord blood (r_s =0,66; p=0.005, and r_s =0.65; p=0.004, respectively).

Discussion

Early-onset neonatal sepsis is associated with high mortality and many adverse consequences for the child's further development. Neonatologists' fear of reacting too late to symptoms that may suggest infection, in the absence of a sufficiently sensitive and specific marker to differentiate sepsis from other acute conditions, influences the decisions of prompt antibiotic prescription, often unnecessarily. The negative influence of antibiotic therapy on the intestinal microbiota and the developing immune system of the newborn is emphasized [20].

In our study, the concentration of lipopolysaccharide-binding protein (LBP) and neutrophil elastase in the cord blood plasma of full-term and premature newborns with symptoms of sepsis were assessed. Higher LBP concentrations are already found in the umbilical cord blood of newborns who develop symptoms of infection in the first days of life, both term and premature babies. This is consistent with reports by other authors. However, there aren't too many published works that tested the concentration of LBP in the umbilical cord blood.

Chen et al. [21] investigated the usefulness of LBP in the diagnosis of intraamniotic infections. They determined the level of LBP in the plasma of women who gave birth not earlier than 24 hours after the departure of the amniotic fluid. Higher levels of LBP were found in mothers whose children were admitted to the intensive care unit due to suspected congenital infection and in the case of histologically confirmed intraamniotic infection. Espinoza et al. [22] found elevated LBP levels in the amniotic fluid in preterm labor and

intraamniotic infection. Roos et al. [23] examined the concentration of LBP in the amniotic fluid and umbilical cord blood. In these studies, the increased level of LBP in the amniotic fluid was associated with the elevated level of cytokines: TNF-α, IL-6, IL-8, maternal fever > 38 ° C, but appeared also in women during labor without diagnosed infection, while an increase in LBP in umbilical cord blood was observed in histologically confirmed chorioamninitis.

Berner et al. [24] found elevated LBP levels in umbilical cord and venous blood collected shortly after delivery in neonates with sepsis.

Elevated LBP levels have been reported in children with severe congenital heart disease, Kawasaki disease, and haemolytic uremic syndrome [25-27]. Behrendt et al. [28] demonstrated the ability of premature babies over 28 weeks of gestation to produce LBP. Pavcnik-Arnol et al. [29] proposed a LBP cut-off point in the umbilical cord blood > 11.97 μg/ml, with a specificity of 91.14% and a sensitivity of 70.83% in preterm newborns, and 98.57% and 80% respectively in newborns born on time. Our results are almost completely consistent.

Granulocyte elastase was tested in a population of newborns since the 1990s, it has not found a consistent place among the routinely used markers of infection.

Tsaka and Herkner [30] found an increase in the activity of neutrophilic elastase in full-term and premature newborns with sepsis and localized infections (pneumonia, urinary tract infection, pyelonephritis). They did not observe an increase in elastase in viral infections. Tegtmeyer et al. [31] investigated the level of neutrophilic elastase in 74 newborns within the first 3 days of the onset of clinical symptoms of sepsis. They found that elastase values were elevated at the onset of clinical sepsis symptoms in 94%, while CRP was only increased in 54% of the newborns tested. Similar results were achieved by Jensen et al. [32]. According to these authors, the increase in neutrophilic elastase in infected newborns was more frequent at the onset of clinical manifestation of infection (in 75% of newborns) compared to CRP (increase in 44% of newborns), and similarly to the leukocyte index (increase in 76% of newborns), both in early and late onset sepsis. In 3 cases, he observed an increase in elastase despite neutropenia.

In obstetrics Kidokoro et al. [33] found an increase in the concentration of neutrophilic elastase in the amniotic fluid in histologically confirmed chorioamnionitis.

Hata et al. [34] assessed the concentration of IL 6, IL-8 and neutrophilic elastase in umbilical cord blood serum in a situation of fetal risk. They found significantly higher

concentration of elastase in the group of neonates with intrauterine growth restriction and fetal risk.

The usefulness of granulocytic elastase as an early marker of sepsis was investigated in children [35,36]. Laskowska-Klita et al.[37] investigated the level of elastase in the cord blood of healthy and infected newborns, full-term and premature babies. They found a significantly higher level of elastase in full-term infected infants compared to healthy ones. In preterm newborns, both infected and asymptomatic, this level was significantly lower than in full-term neonates. They associated it with decreased activity of neutrophils and their decreased ability to phagocytose, as well as decreased ability to release proteinases in preterm babies. Similar conclusions were drawn by Henneke et al.[38]. According to these authors, polynuclear granulocytes in preterm newborns have impaired ability to respond to LPS stimulation.

Obstetric and neonatal complications may increase the concentration of elastase. Rodwell et al.[39] observed an increase in the concentration of elastase in neonates with severe perinatal hypoxia, in the course of serological conflict in the ABO system, in embryopathy resulting from cytomegalovirus infection. They also found increased values in newborns of mothers with pregnancy-induced hypertension and in newborns with TTN (transient tachypnea of newborns). According to these authors, elastase is a sensitive but not specific indicator of infection. Similar conclusions were made by Speer et al.[35]. They observed an increase in neutrophilic elastase in sepsis, but also in localized infections such as pneumonia, enteritis, meconium aspiration syndrome, prolonged acidosis, and persistent fetal circulation. In contrast, elastase remained at a low level in the infant respiratory distress syndrome.

In our study, a comparative analysis of the concentrations of neutrophilic elastase in the cord blood plasma showed significantly higher concentrations in the groups of full-term or premature newborns with systemic infection compared to the control groups. Sampériz et al.[40] investigated the concentration of neutrophilic elastase in the umbilical blood in newborns born between 30 and 42 weeks of gestation. They found significantly higher concentrations of elastase in the umbilical cord blood in newborns with probable or confirmed maternal-fetal infection.

The subject of our research was also to assess the correlation of neutrophilic elastase with routinely used in clinical practise laboratory markers of infection. In the group of newborn infants with infection, a positive correlation was demonstrated between the concentrations of neutrophilic elastase and CRP in the umbilical cord blood. Tegtmeyer et al.[31] investigated the concentration of neutrophilic elastase in early and late-onset sepsis. In

these studies, the increase in the concentration of neutrophilic elastase correlated with the increase in CRP, but was much ahead of it.

Limitations of the study. The limitation of our study is the difficulty of obtaining homogeneous groups of patients with an appropriate size.

Conclusions

Measuring the concentration of LBP and neutrophil elastase in cord blood plasma may help in the detection and prediction of early onset sepsis in newborns with prenatal risk factors. Cord blood LBP is a highly specific and sensitive biomarker of sepsis immediately after birth. High levels of LBP and neutrophil elastase in cord blood correlate with CRP.

Declaration of Conflicting Interests: none.

According to the Helsinki Declaration: Ethical Approval/Patients consent: Bioethics Committee (BN-001/15/05)

Funding Statement: Statutory activities of the university

Author contributions: Conceptualization: A.K., Methodology: A.K., A.B., Software: J.K., Validation: A.B., Formal Analysis: A.K., A.B., Investigation: A.B., Resources: A.B., J.K.
Data Curation: A.B.; Writing – original draft preparation: A.B.; Writing – Review and Editing: A.K., A.B.,
All authors have read and agreed with the published version of the manuscript.

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