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Abstract: (1) Background: Neonatal early-onset sepsis (EOS) is associated with important mortality and morbidity. The aims of this study were to evaluate the association between serum biomarkers with early onset neonatal sepsis in a cohort of patients with prolonged rupture of membranes (PROM), and to calculate their diagnostic accuracy. (2) Methods: A retrospective cohort study was conducted on 1355 newborns with PROM admitted between January 2017 and March 2020, who were divided in two groups: group A, with PROM ≥ 18 hours and group B, with PROM < 18 hours. Both groups were further split into subgroups: proven sepsis, probable sepsis and no sepsis. Descriptive statistics, ANOVA analysis, and a Random Effects Generalized Least Squares (GLS) regression were used to evaluate the data. (3) Results: The statistically significant predictors of neonatal sepsis were the high white blood cells count from the first ($p = 0.005$) and third day ($p = 0.028$), and high CRP values from the first day ($p = 0.004$). Procalcitonin (area under the curve - AUC = 0.78) and CRP (AUC = 0.76) measured on the first day had the best predictive performance for early onset neonatal sepsis. (4) Conclusions: Further studies are needed to explore other biomarkers of sepsis in both pregnant women and newborns to better predict EOS.

Keywords: early-onset sepsis; biomarker; C-reactive protein; procalcitonin; white blood count; antibiotics

1. Introduction

Prolonged rupture of membranes (PROM) occurs in 8-10% of pregnancies [1,2] and may be complicated by microbial invasion of the amniotic cavity, inducing histological chorioamnionitis, intraamniotic inflammation, premature birth and neonatal infection [3]. Approximately one third of spontaneous preterm births are associated with premature prolonged rupture of membranes (PPROM) [4]. Other causes of preterm birth include multiple pregnancies, congenital abnormalities,

chronic maternal conditions (diabetes, hypertension, autoimmune disorders, abdominal or uterine tumors, etc), in vitro fertilization (IVF), extremes of ages or weight, maternal use of illicit drugs or alcohol, psychiatric comorbidities or current pregnancy complications that require an iatrogenic preterm birth [5–15]. Previous studies have proven that the lower the gestational age (GA) at which the membranes rupture, the greater the probability of infection [16,17].

Obstetricians need to choose between conservative management, that could lead to chorioamnionitis and neonatal early-onset sepsis (EOS), and premature delivery, that is associated with prematurity complications. EOS was defined as an infection due to organisms acquired before and during delivery, occurring in the first 72 hours of life [18]. However, there are studies which extend the definition of EOS determined by *group B Streptococcus* (GBS) up to 7 days of life [19–21].

EOS is a challenge for neonatologists, being an invasive infection often suspected, but rarely diagnosed, with a proven diagnosis of 0.8 in 1000 births [22]. Due to significant mortality and severe complications associated with neonatal sepsis, early initiation of broad-spectrum antibiotics is the first step to decrease the morbidity and mortality [23,24]. Positive blood culture is the only certain diagnostic tool, but its results are confirmed within a 36-48 h time frame. Even in the presence of specific signs and symptoms, less than 1% of newborns with suspected sepsis have a positive blood culture [25].

In order to aid in the prompt detection and precise diagnosis of neonatal sepsis, more modern molecular approaches and nonculture-based techniques are required. Although the white blood cells (WBC), the immature/total neutrophils ratio (I/T), and the number of platelets (PLT) do not show high sensitivity and specificity to diagnose infections, these markers are the most used in neonatal units [26].

Leucopenia (WBC count 5000/mm3) has a high specificity (91%), but a low sensitivity (29%) for the diagnosis of newborn sepsis according to a literature review by Sharma et al [27]. I/T ratio may be the most accurate predictor of neonatal sepsis when compared to other hematological indicators, with a value greater than 0.27 in term newborns, and greater than 0.22 in preterm newborns being indicative of neonatal sepsis, but the serum level of this biomarker fluctuates with gestational age and postnatal age [28].

It has been shown that serial C-reactive protein (CRP) measurements increase its sensitivity and negative predictive value for neonatal sepsis, and may be beneficial for assessing the treatment response of affected neonates under antibiotic therapy [29]. Procalcitonin (PCT) could also be considered as a promising biomarker for neonatal sepsis due to high sensitivity (81%; 95% CI: 74–87%) and specificity (79%; 95% CI: 69–87%) values, as reported in a meta-analysis [30].

Other literature data indicated that currently determined markers in the newborns' serum may be elevated due to other factors unrelated to infection, such as: hypertension or maternal fever, prolonged labor, perinatal asphyxia, meconium aspiration syndrome, respiratory distress syndrome, intracranial hemorrhage or pneumothorax [31,32].

The aims of this study were to evaluate the association between specific serum biomarkers with early onset neonatal sepsis in a cohort of patients with PROM, and to calculate their diagnostic accuracy.

2. Materials and Methods

2.1. Study design

An observational retrospective cohort study was conducted using the database of patients admitted to a level III neonatal center " Cuza voda" Obstetrics- Gynecology Clinic Hospital between January 2017 and March 2020. The medical charts of the neonates were retrospectively reviewed. Information on the gestational age, weight, gender, mode of delivery, need for resuscitation, Apgar score, risk factors for infection and clinical signs of sepsis were extracted.

Access to patient's medical records and the study protocol was approved by the Institutional Ethics Committee of the regional hospital (No 5332/21.05.2020). Newborns' personal data were anonymized prior to analysis.

2.2. Definitions and study population

A total of 1355 medical records of neonates were analyzed. Newborns with PROM and postnatal age < 24 hours were included in the study. The gestational age (GA) ranged from 23 to 43 weeks. Exclusion criteria were: infants born at less than 23 weeks of gestation, infants with congenital anomalies, the absence/incomplete sepsis screening according to the unit protocol.

The population was divided in 2 groups. Group A (n=826 patients) included neonates with PROM longer than 18 hours before birth, while the infants with PROM less than 18 hours were assigned to group B (n=529 patients). The cut-off of 18 hours was chosen in accordance to our local protocols.

For the secondary analysis, we further stratified the neonates into 3 subgroups: proven EOS (subgroups A1 and B1), probable EOS (subgroups A2 and B2) and absence of EOS–control group (subgroups A3 and B3). Assignation of patients in one of the subgroups was performed according to the criteria proposed by the European Medicines Agency (EMA) [33].

The suspicion of infection was assessed on admission in all newborns with clinical signs suggesting infection: 1. respiratory signs: apnea, tachypnea, retractions, need for supplemental oxygen/respiratory support; 2. cardio-circulatory signs: tachycardia/bradycardia, hypotension, or impaired peripheral perfusion (mottled skin, cold extremities);   3. oliguria (urine output < 1 ml/kg/h); 4. temperature instability, hypothermia or hyperthermia; 5. gastrointestinal signs: vomiting, abdominal distension, bilious/bloody gastric aspirates; 6. skin and subcutaneous signs: petechial rash or scleroderma; 7. neurological signs: irritability, lethargy, hypotonia, weak sucking.

The sepsis panel included: complete blood count (CBC), C-reactive protein (CRP), procalcitonin (PCT), fibrinogen and blood culture, that was performed at admission, at 72 hours of life and on the 5th day of life, except for the blood culture and procalcitonin levels that were determined on a single occasion, at admission. Serum parameters were flagged as abnormal as follows: leukopenia (< 4 x10^9 cells/L) or leukocytosis (> 20 x 10^9 cells/L), immature to total neutrophils ratio (I/T) > 0.2, thrombocytopenia (< 100 x 10^9 cells/L) and inflammatory syndrome (CRP > 10 mg/L or PCT ≥ 10 ng/mL). According to the local protocol, antibiotic therapy was started in all neonates with clinical suspicion of infection and/or risk factors for sepsis (including rupture of membranes).

An amniotic fluid culture was performed for all pregnant patients with ruptured membranes before delivery. Clinical chorioamnionitis was diagnosed in the presence of maternal fever with two of the following: maternal tachycardia, fetal tachycardia, uterine tenderness, foul odor of amniotic fluid, or maternal leukocytosis [34]. Leukocytosis (> 10.000/mm^3) and high CRP values (> 6 mg/dL) were considered as signs of maternal inflammatory syndrome according to the local laboratory thresholds. Maternal fever was considered when the core body temperature was higher than 38° Celsius.

2.3. Statistical analysis

The paired sample t-test and independent-sample t-test were used for continuous variables. Continuous variables were presented as the mean +/- standard deviation (SD). Categorical variables were presented as frequencies with corresponding percentages.

ANOVA analysis with the Bonferroni post-hoc test was used to determine whether or not there is a statistically significant difference between the means of serum biomarkers (WBC, CRP, and fibrinogen) between subgroups, and boxplots were used for graphical representations of these differences.

A Random Effects Generalized Least Squares (GLS) regression was used to measure the association between predictor variables, such as serum biomarkers levels (WBC, CRP, and fibrinogen) measured on three different occasions, and an outcome variable, proven neonatal sepsis. The performance of laboratory biomarkers in the diagnosis of EOS was calculated by using area under the curve-receiver operating curve (AUC–ROC). These analyses were performed using STATA SE (version 15, StataCorp LLC). A p-value of less than 0.05 was considered statistically significant.

3. Results

According to the criteria mentioned, 826 newborns were assigned to the group with PROM ≥ 18 hours, out of which 10 were included in the proven EOS group (0.7%), 414 neonates in the probable EOS category (30.6%), and 402 in the control group, without sepsis (29.7%).   Another 549 neonates with PROM < 18 hours were segregated into proven EOS category (n= 11 patients, 0.8%), 266 in the probable EOS category (19.6%), and 252 in the control group, without sepsis (18.6%) (Figure 1).
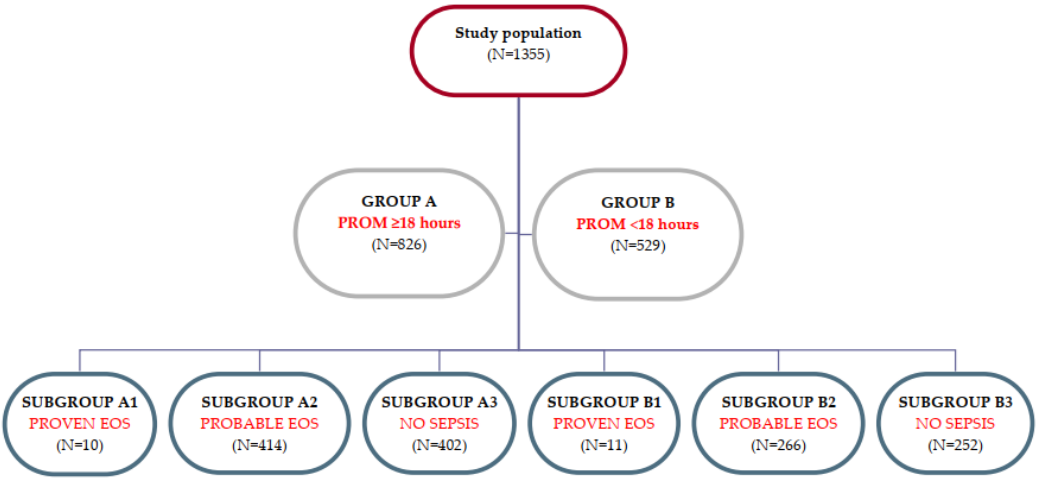

Figure 1. Flowchart of the study group distribution.

A significant difference was found among the groups and each of the subgroups regarding the GA, birth weight (BW), as shown in Tables 2 and 3.

Antibiotics were more frequently administered to neonates in the group with PROM ≥ 18 hours ($p < 0.001$). Duration of hospital stay was significantly longer for patients in group A compared to group B (15.37 ± 21.54 days vs. 7.67 ± 12.98 days, $p < 0.001$).

Table 1. Clinical and demographic characteristics of the study group.

	PROM ≥18 hours (group A) N=826	PROM <18 HOURS (GROUP B) n=529	*P* value
GA mean ± SD	36.15 ± 3.9	37.83 ± 2.528	<0.001
Preterm*	332	86	<0.001
BW mean ± SD	2730.3 ± 857.7	3146.3 ± 636.2	<0.001
CS	371	245	0.615
VB	455	284	0.614
Male	419	307	0.009
Female	407	222	0.009
Apgar 1 min - median	7	8	<0.001
Apgar 5 min - median	8	9	<0.001
Apgar 10 min - median	8	9	<0.001

* Table 1 legend: GA: Gestational Age; BW: Birth Weight; CS: C-section; VB: vaginal birth; *GA < 37 weeks.

The Apgar scores were lower in neonates with proven sepsis (Subgroups A1 and B1) compared to those with probable sepsis or without sepsis, irrespective of duration of ruptured membranes (Table 2).

Moreover, a statistically significant difference was found when comparing the Apgar scores of group A with group B ($p < 0.001$). The proportion of preterm newborns was higher in the proven EOS

group. Prolonged rupture of membranes ≥ 18 hours was associated with longer hospital stay in all subgroups (Table 2).

Table 2. Clinical and demographic characteristics of the newborns in the subgroups.

	Proven EOS (A1) N=10	Proven EOS (B1) N=11	*P* value	Probable EOS (A2) N=414	Probable EOS (B2) N=266	*P* value	No sepsis (A3) N=402	No sepsis (B3) N=252	*P* value
GA (mean ± SD)	29.5 ± 4.3	35.3 ± 3.1	0.002	35.6 ± 4.3	37.6 ± 3.1	<0.001	36.8 ± 3.1	38.1 ± 1.6	<0.001
Preterm*	9	7	0.157	185	46	<0.001	138	32	<0.001
BW (median)	1484	2636	0.005	2605	3109	<0.001	2889	3250	<0.001
CS	4	8	0.130	188	123	0.519	179	114	0.969
VB	6	3	0.130	226	143	0.832	223	138	0.859
Male	6	8	0.014	204	152	0.045	213	147	0.181
Female	4	3	0.016	210	114	0.045	189	105	0.181
Apgar 1 min (median)	6	7	0.475	8	8	<0.001	8	9	<0.001
Apgar 5 min (median)	7	8	0.453	8	9	0.004	9	9	<0.001
Apgar 10 min (median)	7	8	0.413	8	9	0.006	9	9	<0.001
Duration of stay (mean ± SD)	52.10 ± 40.5	22.73 ± 12.1	<0.005	18.80 ± 24.6	9.75 ± 17.1	<0.001	10.92 ± 15.1	4.81 ± 3.8	<0.001

*Table 2 legend: GA: Gestational Age; BW: Birth Weight; CS: C-section; VB: vaginal birth; *GA < 37 weeks.

Analysis of perinatal risk factors for EOS revealed that the number of positive amniotic fluid cultures was significantly higher in group A (p = 0.001) compared to group B. Subgroup analysis revealed that positive amniotic fluid culture was associated only with probable EOS (p = 0.001), but no statistical significance was found between subgroups A1/B1 or A3/B3.

Furthermore, 12 of the mothers in subgroup A2 had chorioamnionitis, compared to none in B2 subgroup (p = 0.005). Also, there were no statistically significant differences concerning other analyzed risk factors such as foul-smelling amniotic fluid, maternal fever or maternal inflammatory syndrome between neonates in either subgroup (Table 3).

Table 3. Perinatal risk factors for infection.

	Proven EOS (A1) N=10	Proven EOS (B1) N=11	*P* value	Probable EOS (A2) N=414	Probable EOS (B2) N=266	*P* value	No sepsis (A3) N=402	No sepsis (B3) N=252	*P* value
Positive amniotic fluid culture	5	0	0.007	58	15	0.001	19	8	0.83
Foul smelling amniotic fluid	2	3	0.28	41	27	0.41	16	16	0.739
Maternal fever	1	1	0.94	6	3	0.72	1	0	0.428
Maternal inflammatory markers	0	0	-	5	6	0.29	1	0	0.428

*Table 3 legend: EOS- early onset sepsis.

Regarding neonatal complications, respiratory distress syndrome (RDS) and retinopathy of prematurity (ROP) were significantly more frequent in group A than in group B (p < 0.001).

Pneumothorax and pulmonary hemorrhage were statistically significant in subgroup A2 compared to group B2 (p = 0.002). Complications including mortality and patients included in the study are shown in Table 5. One neonate in subgroup A1 died due to EOS with Staphylococcus capitis (p = 0.28).

When comparing subgroup A1 with B1 more infants required mechanical ventilation (50% vs 27%; p = 0.806), for a longer period (13.8 vs 3.6 days; p = 0.322). However, no statistical significance was found. Inotropic support was necessary for 3 infants (30%) of subgroup A1, and 2 infants (18.2%) of subgroup B1 (p = 0.525).

Table 4. Neonatal mortality and complications in the studied subgroups.

	Proven EOS (A1) N=10	Proven EOS (B1) N=11	*P* value	Probable EOS (A2) N=414	Probable EOS (B2) N=266	*P* value	No sepsis (A3) N=402	No sepsis (B3) N=252	*P* value
	Short term complications								
RDS	9	7	0.157	134	57	0.002	68	5	<0.001
PPHN	1	0	0.28	7	7	0.399	2	0	0.262
Pulmonary hemorrhage	0	1	0.329	1	1	0.002	1	0	0.428
Pneumothorax	1	0	0.366	8	4	0.002	2	2	0.636
Severe IVH*	0	1	0.32	8	6	0.772	0	0	-
	Long term complications								
NEC	1	0	0.28	4	0	0.049	0	0	0.428
ROP	2	0	<0.001	24	4	0.006	4	0	0.112
BPD	0	1	0.329	6	4	0.954	4	0	0.428
Antibiotherapy	9.2	10.7	0.801	4.6	8	0.501	8	5.8	0.562
Duration of stay Mean ± SD	52.10 ± 40.5	22.73 ± 12.1	<0.005	18.80 ± 24.6	9.75 ± 17.1	<0.001	10.92 ± 15.1	4.81 ± 3.8	<0.001
Mortality	1	0	0.28	8	7	0.54	7	0	0.428

* Table 4 legend: RDS: Respiratory distress syndrome; PPHN: persistent pulmonary hypertension of the newborn; IVH: intraventricular hemorrhage; NEC: necrotizing enterocolitis; ROP: retinopathy of prematurity; BPD: bronchopulmonary dysplasia; severe IVH* > grade III; EOS- early onset sepsis; SD- standard deviation.

Of the total 1355 patients, microorganisms were identified in the blood samples of 21 neonates (subgroup A1: 10 infants, and subgroup B1: 11 infants). The most common pathogen responsible for EOS was Staphylococcus spp. (n= 6; 28%; p = 0.89). Among neonates with EOS and PROM ≥18 hours, Escherichia coli was the most frequently detected pathogen (n=5; 50%), whereas in group B, the most prevalent pathogen was Klebsiella pneumoniae (n=4; 36.3%) (Table 5).

Table 5. Comparison of blood culture results from the neonates with PPROM.

Microorganism	PROM ≥18 hours %	PROM <18 HOURS %	Total (%)
Staphylococcus spp.	3 (30)	3 (27.3)	6 (28.5)
Klebsiella pneumoniae	1 (10)	4 (36.4)	5 (23.8)
Escherichia coli	5 (50)	0	5 (23.8)
Streptococcus spp.	1 (10)	3 (27.3)	4 (19.2)
Listeria monocytogenes	0	1 (9)	1 (4.7)

* Table 5 legend: PROM- prolonged rupture of membranes.

A comparison of hematological parameters between groups is summarized in Table 6. Significant leukocytosis and high CRP values measured in day 1 were encountered in group B, with PROM <18 hours (p < 0.001).

Table 6. Comparison of the hematological parameters of the groups.

Parameters	PROM >18 hours GROUP A			PROM <18 hours GROUP B			Independent t-Test	
	Mean ± SD			Mean ± SD			T	*P* value
WBC x 10^3 /mm³ D1/D3/D5	**19.54 ± 8.02**	**14.45 ± 8.21**	**13.1 ± 6.34**	21.2 ± 7.87	14.34 ± 6.54	13.19 ± 7.61	-3.7 0.2 0.0	<0.001 >0.05 >0.05
I/T ratio	0.14 ± 0.11			0.15 ± 0.08			-0.53	>0.05
CRP mg/L D1/D3/D5	10.83 ± 9.56	11.86 ± 11.26	8.58 ± 15.06	13.02 ± 12.4	12.97 ± 18.35	8.51 ± 9.40	-3.3 -1.0 0.0	<0.001 >0.05 >0.05
Fibrinogen D1/D3/D5	270 ± 98.3	332 ± 118.6	362 ± 88.3	268 ± 79.8	362± 114.2	340 ± 108.9	0.16 -1.7 1.4	>0.05 >0.05 >0.05

* Table 6 legend: PROM- prolonged rupture of membranes; WBC- white blood cells; CRP- c- reactive protein; I/T- immature/total neutrophils ratio; D- day; SD- standard deviation.

Table 7 summarizes the descriptive statistics and t-tests for the main serum biomarkers with repeated measurements among subgroups with proven EOS. However, none of the evaluated parameters were significantly different between A1 and B1 subgroups.

Table 7. Comparison of the serum biomarkers measurements for the proven sepsis subgroups.

PROVEN SEPSIS Subgroup A1 Subgroup B1	Day 1	Day 3	Day 5	Independent t-test	
	Mean ± SD	Mean ± SD	Mean ± SD	t	*P* value
WBC x 10^3 /mm³	12.05 ± 7.74	13.15 ± 10.89	17.71 ± 6.98	-0.8	>0.05
	15.1 ± 8.03	15.49 ± 8.13	23.67 ± 15	-0.5 -1.0	>0.05 >0.05
I/T ratio	0.20 ± 0.08			2.0	>0.05
	0.12 ± 0.04				
CRP mg/L	21.5 ± 19.10	38.5 ± 18.75	20.64 ± 14.25	0.4	>0.05
	19.57 ± 16.93	38.36 ± 7.56	47.88 ± 6.48	0.0 2.1	>0.05 >0.05
Fibrinogen	399 ± 81.4	450 ± 191	380 ± 103	0.7	>0.05
	355 ± 109.4	457 ± 125	358 ± 104	-0.0 0.3	>0.05 >0.05

* Table 7 legend: WBC- white blood cells; CRP- c- reactive protein; I/T- immature/total neutrophils ratio; SD- standard deviation.

Table 8 summarizes the descriptive statistics and t-tests for the main serum biomarkers with repeated measurements among subgroups with suspected EOS, and without neonatal sepsis. Leukocytosis and CRP serum values recorded in the first day were significantly higher in the B2 subgroup compared to A2 subgroup (*p* < 0.05). Also, when taking into consideration the subgroups without neonatal sepsis, our results showed that leukocytosis on day 1, CRP on day 3, and fibrinogen

levels on day 2 were significantly higher in the B3 subgroup compared to A3 subgroup ($p < 0.05$). The procalcitonin serum levels were significantly higher (cut-off 10 ng/ml) in the B2 subgroup compared to A2 subgroup ($p < 0.05$), while for other subgroups it did not differ significantly.

Table 8. Comparison of the serum biomarkers measurements for the subgroups with suspected sepsis or without proven sepsis.

Suspected EOS Subgroup A2 Subgroup B2	Day 1 Mean ± SD	Day 3 Mean ± SD	Day 5 Mean ± SD	Independent t-test t	P value
WBC x 10^3 /mm³	20.13 ± 9.15	13.92 ± 9.15	15.31 ± 9.05	-2.4	<0.05
	21.9 ± 9.18	13.23 ± 6.9	14.83 ± 7.09	0.6	>0.05
				0.7	>0.05
I/T ratio	0.14 ± 0.12			-0.4	>0.05
	0.15 ± 0.09				
CRP mg/L	13.96 ± 10.17	13.4 ± 6.7	17.6 ± 9.78	-3.0	<0.05
	17 ± 4.37	13.8 ± 9.32	18 ± 10.7	-0.3	>0.05
				0.2	>0.05
Fibrinogen	277 ± 104	343 ± 120	364 ± 87.6	0.1	>0.05
	275 ± 84.7	344 ± 109	342 ± 110	-0.0	>0.05
				1.3	>0.05
No sepsis Subgroup A3 Subgroup B3	Day 1 Mean ± SD	Day 3 Mean ± SD	Day 5 Mean ± SD	t	P value
WBC x 10^3 /mm³	19.09 ± 6.44	13.02 ± 6.13	11.42 ± 4.80	-3.0	<0.05
	20.76 ± 5.57	13.39 ± 5.19	10.55 ± 3.99	-0.5	>0.05
				0.9	>0.05
I/T ratio	0.104 ± 0.09			-3.5	>0.05
	0.14				
CRP mg/L	7.03 ± 6.5	7.18 ± 5.77	4.99 ± 3.38	-0.9	>0.05
	7.57 ± 5.6	7.80 ± 6.93	6.22 ± 3.42	-0.7	>0.05
				-2.0	<0.05
Fibrinogen	257 ± 86	293 ± 92.67	350 ± 88	0.1	>0.05
	255 ± 68.11	383 ± 99.59	320 ± 115	-2.5	<0.05
				0.8	>0.05

* Table 8 legend: EOS- early onset sepsis; WBC- white blood cells; CRP- c- reactive protein; I/T- immature/total neutrophils ratio; SD- standard deviation.

ANOVA analysis of variance along with the Bonferroni post-hoc multiple comparisons (Table 9) and boxplots (Figures 2–4) were used for explanatory analysis of the differences in serum biomarkers concentrations (CRP, WBC, and fibrinogen) between subgroups. While ANOVA analysis revealed in the majority of cases a statistically significant difference in mean serum biomarkers concentrations between at least two groups (p < 0.05), the Bonferroni post-hoc test found that a statistically significant mean difference in: white blood cells count from the first day between A2-B2 (p= 0.010; 95% CI: -16.24- -1.17), and A3-B3 subgroups (p= 0.022; 95% CI: -15.53- -0.64), serum CRP concentration from the first day between A2-B2 subgroups (p = 0.003; 95% CI: -5.47- -0.64), serum CRP concentration from the fifth day between A3-B3 subgroups (p = 0.04; 95% CI: 10.27-39.14), and serum fibrinogen concentration from the third day between A2-B2 subgroups (p = 0.008; 95% CI: 0.86-10.36), respectively.

Table 9. ANOVA analysis of variance and Bonferroni post-hoc test for the serum biomarkers for neonatal sepsis.

Serum biomarkers	Groups	ANOVA results		Bonferroni test				
		F score	*P* value	Mean difference	Standard error	*P* value	95% confidence interval lower limit	95% confidence interval upper limit
WBC day 1	A1-B1	7.13	<0.001	-3.05	3.45	1.000	- 13.21	7.11
	A2-B2			-8.71	2.56	0.010	-16.24	-1.17
	A3-B3			-8.08	2.53	0.022	-15.53	-0.64
WBC day 3	A1-B1	2.79	0.016	-2.34	3.59	1.000	-12.92	8.24
	A2-B2			0.48	0.68	1.000	-1.53	2.50
	A3-B3			-0.037	0.90	1.000	-3.03	2.28
WBC day 5	A1-B1	8.27	<0.001	-5.96	3.23	0.98	-15.49	3.57
	A2-B2			0.68	0.82	1.000	-1.73	3.10
	A3-B3			0.87	1.28	1.000	-2.91	4.66
CRP day 1	A1-B1	41.43	<0.001	1.92	4.92	1.000	-12.55	16.40
	A2-B2			-3.05	0.82	0.003	-5.47	-0.64
	A3-B3			-0.54	0.91	1.000	-3.22	2.13
CRP day 3	A1-B1	21.4	<0.001	0.13	6.33	1.000	-18.50	18.78
	A2-B2			-0.39	1.16	1.000	-3.84	3.05
	A3-B3			-0.61	1.79	1.000	-5.91	4.68
CRP day 5	A1-B1	3.2	0.007	11.19	6.37	1.000	-7.59	29.98
	A2-B2			0.45	1.41	1.000	-3.72	4.62
	A3-B3			24.70	4.90	0.04	10.27	39.14
Fibrinogen day 1	A1-B1	5.2	<0.001	43.9	55.08	1.000	-118.29	206.09
	A2-B2			1.41	9.02	1.000	-25.16	27.98
	A3-B3			1.34	10.40	1.000	-29.28	31.97
Fibrinogen day 3	A1-B1	4.96	<0.001	-7.19	63.63	1.000	-195.52	181.14
	A2-B2			5.60	1.61	0.008	0.86	10.36
	A3-B3			-90.39	42.92	0.541	-217.45	36.65
Fibrinogen day 5	A1-B1	0.73	0.59	21.16	50.95	1.000	-130.06	172.39
	A2-B2			22.34	16.88	1.000	-27.75	72.44
	A3-B3			-1.23	2.34	1.000	-8.14	5.68

* Table 9 legend: WBC- white blood cells; CRP- c- reactive protein; I/T- immature/total neutrophils ratio.
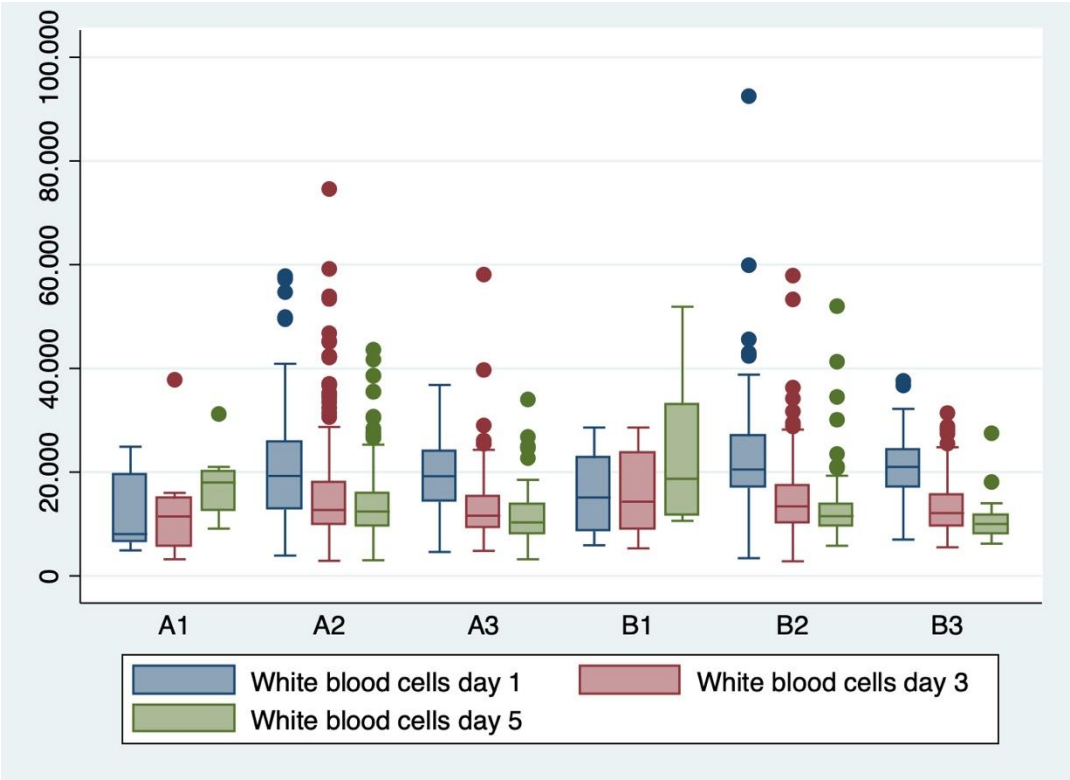
Figure 2. Boxplot representing the white blood cells count from days 1, 3, and 5 in the evaluated subgroups (A1-A3, B1-B3).
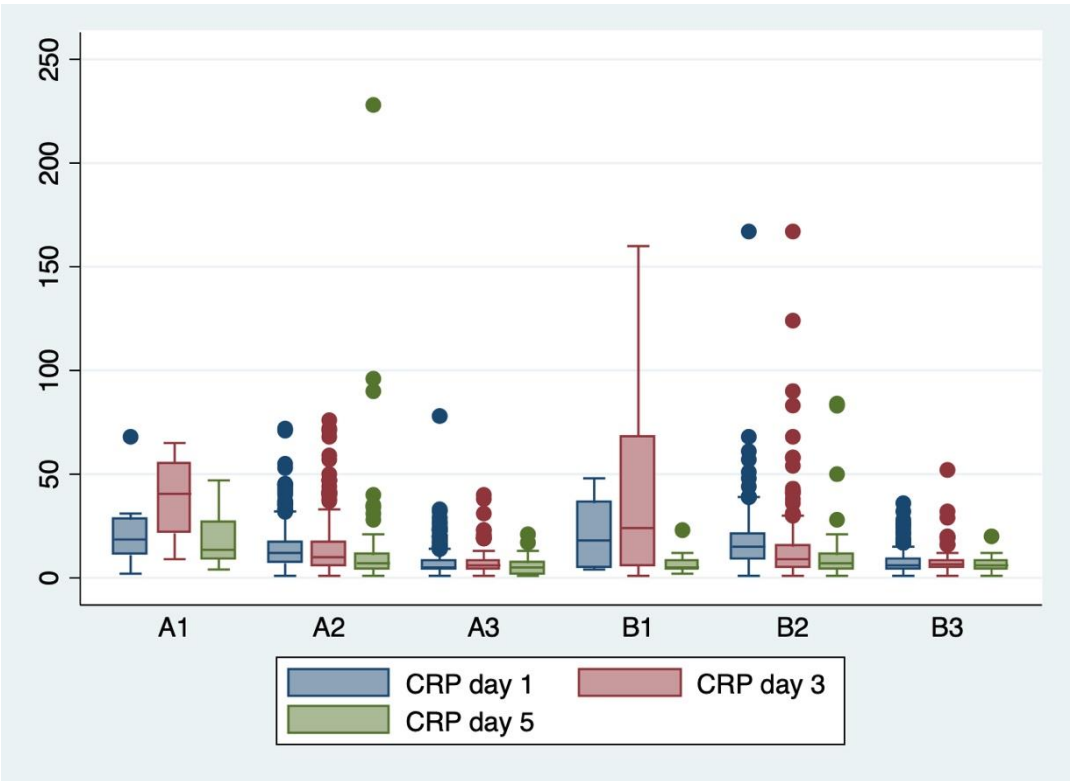

Figure 3. Boxplot representing the C-reactive protein (CRP) serum concentrations from days 1, 3, and 5 in the evaluated subgroups (A1-A3, B1-B3).
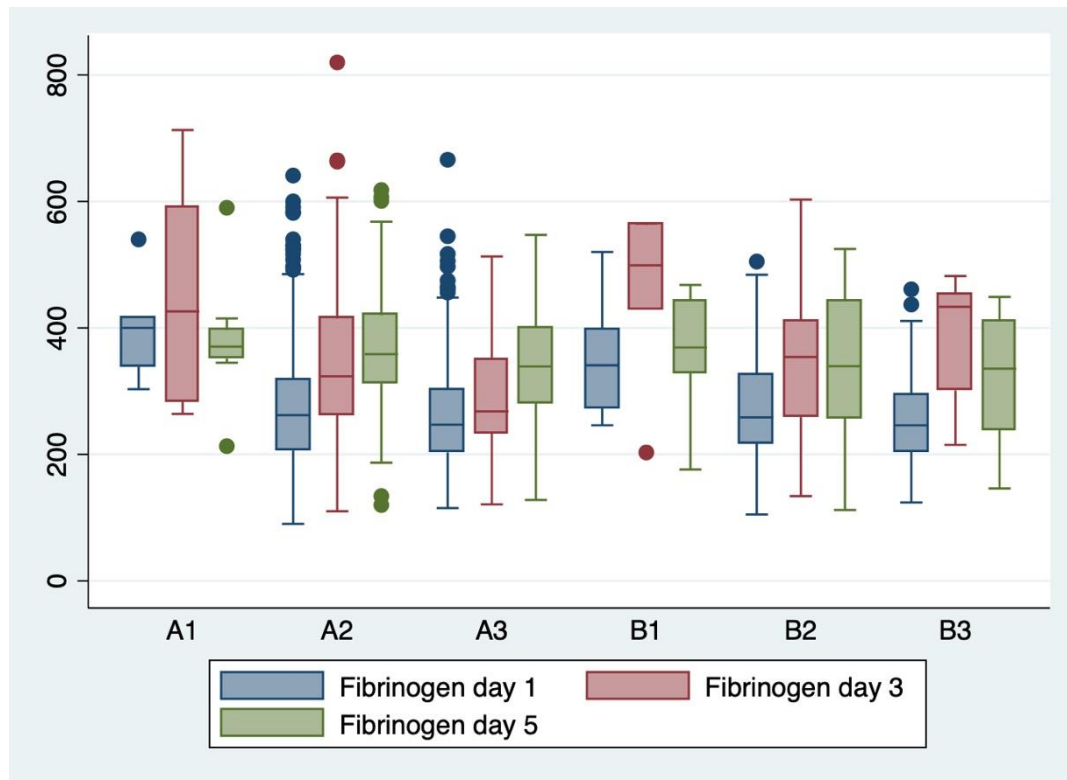
Figure 4. Boxplot representing the fibrinogen serum concentrations from days 1, 3, and 5 in the evaluated subgroups (A1-A3, B1-B3).

A Random Effects GLS regression was used to measure the association between predictor variables, such as serum biomarkers levels (WBC, CRP, and fibrinogen) measured on three different occasions, and an outcome variable, proven neonatal sepsis (Table 10). Our model had a *p value* < 0.001, a R-squared value within groups of 0.47, and between groups of 0.82, while rho was 0.42. The statistically significant predictors of neonatal sepsis were the white blood cells count from the first (p = 0.005) and third day (p = 0.028), and CRP values from the first day (p = 0.004).

Table 10. Random Effects Random Effects Generalized Least Squares regression of biochemical predictors for neonatal sepsis.

Serum biomarkers	*P value*	95% Confidence interval
WBC day 1	0.005	0.016- 0.09
WBC day 3	0.028	-0.08- -0.004
WBC day 5	0.769	-0.04- 0.03
CRP day 1	0.004	-0.01- 0.02
CRP day 3	0.063	-0.0008- 0.03
CRP day 5	0.483	-0.044- 0.021
Fibrinogen day 1	0.642	-0.002- 0.001
Fibrinogen day 3	0.823	-0.002- 0.002
Fibrinogen day 5	0.117	-0.004- 0.0004

* Table 10 legend: WBC- white blood cells; CRP- c- reactive protein.

Table 11 summarizes the AUC for proven sepsis of the WBC, CRP, and fibrinogen serum levels measured on three different occasions, as well as for I/T and procalcitonin measured on one occasion. Moreover, it summarizes the AUC values corresponding to various combinations of these biomarkers. Our results showed that procalcitonin (AUC - 0.78) and CRP measured on the first day (AUC - 0.76) had the best predictive performance for early onset neonatal sepsis. The best predictive performance for this type of sepsis was obtained by the combinations of biomarkers: WBC, CRP, and

fibrinogen recorded on the first day (AUC: 0.83), and WBC, CRP, and fibrinogen recorded on the third day (AUC: 0.90), respectively. The corresponding plots of AUC are presented as supplementary materials (Figure S1- S15).

Table 11. AUC for proven sepsis.

Biomarker	AUC value
WBC day 1	0.55
WBC day 3	0.47
WBC day 5	0.49
CRP day 1	0.76
CRP day 3	0.66
CRP day 5	0.62
Fibrinogen day 1	0.34
Fibrinogen day 3	0.37
Fibrinogen day 5	0.35
I/T	0.58
PCT	0.78
WBC+ CRP+Fibrinogen (day 1)	0.83
WBC+ CRP+Fibrinogen (day 3)	0.90
WBC+ CRP+Fibrinogen (day 5)	0.70
I/T+ PCT	0.76

* Table 11 legend: WBC- white blood cells; CRP- c- reactive protein; I/T- immature/total neutrophils ratio; PCT-procalcitonin.

4. Discussion

In order to prevent the morbidity and mortality related to EOS, identifying the main risk factors along with early diagnosis and therapy are crucial. Along with PROM, GA <30 weeks, male sex, birth weight <1500 g, inadequate prenatal care, low socio-economic status of the mother, poor maternal nutrition, maternal substance abuse, clinical chorioamnionitis and lack of intrapartum antibiotics were cited as risk factors for neonatal sepsis [18,35].

Our study revealed that prematurity was significantly more frequent in the EOS group (p<0.001). We found that out of 16 (76%) preterm neonates who developed EOS, 9 patients (90%) had PROM ≥ 18 hours, and 7 patients (63.7%) had PROM < 18 hours. Consistent with the data reported in the literature, the present study also showed that in the subgroup with proven sepsis male infants were predominant (subgroup A1: n=6, 60%; subgroup B1: n=8, 72%, EOS subgroups: n=14, 66%), although this parameter did not present statistical significance [36–39]. Male neonates were reported to be at higher risk for EOS, according to the study on the largest population (n=56 261, 50.8%) and the one extended over the longest period (18 years) [39,40]. However, there are studies whose results have identified an equal gender ratio or a predominance of females [41–43].

Marks et al. reviewed the literature over a period of 40 years in order to estimate the time until obtaining positive blood cultures in EOS [44]. On a total of 6188 blood cultures (5848 neonates), 250 positive cultures were identified, of which 146 were contaminants. The majority of blood cultures (54%) were positive for Group B Streptococcus (GBS). Moreover, in their study, 7 out of 8 (20%) neonates positive to Escherichia coli died from EOS. Recent studies, identified a change in the distribution of organism causing EOS, with a predominance of Gram-negative rods, especially Escherichia coli [45]. National Institute of Child Health and Human birth (NICHD), identified an increase from 3.2 to 5.09/1000 in Escherichia coli EOS and a decrease in GBS sepsis from 5.9 to 2.08/1000 on a cohort of VLBW infants [46].

Our results were consistent with these changes, Escherichia coli (n= 5/10; 50%) being the agent most isolated in group A, followed by Staphylococcus spp. (n= 3/10; 30%), Klebsiella pneumoniae (n= 1/10; 10%) and Streptococcus spp. (n= 1/10; 10%). In contradiction to group A, the pathogens inducing

sepsis in group B were Klebsiella pneumoniae (n=4/11; 36.3%), Staphylococcus spp. (n= 3/11; 27.2%), Streptococcus spp. (n= 3/11; 27.2%) and Listeria monocytogenes (n=1/11; 9%). Sabry et al., identified Klebsiella pneumoniae as the leading cause of EOS in term infants [47]. In another study on term infants EOS was most frequently caused by Group B Streptococcus, Escherichia coli and Enterococcus spp. [48]. By comparison, the present study showed the following bacteria on term infants: group A (Staphylococcus epidermidis: n=1, 10%), whereas in group B there were 2 neonates with Klebsiella pneumoniae (27%), 1 with Staphylococcus epidermidis (18%), and 1 was positive for both of them. The rate of gram-negative infection was higher in term than in preterm infants (60% vs 45%). In contrast, the rate of gram-positive sepsis was higher in preterm than in term infants (76% vs 24%). Overall, gram-positive organisms were isolated in 12 patients (57%), and gram-negative pathogens were identified in the probes of 9 infants (42%).

However, there are still numerous studies which reported Group B Streptococcus to be the most prevalent cause of EOS, especially in term infants [49,50]. These also include studies conducted in Southern Europe [51]. In the Greek study, this happens after the exclusion of Coagulase-negative staphylococci (CoNS) which were predominant (28.6%) [51].

Our region ranked first in the European colonization rate (6–32%), followed by Eastern Europe (19–29%) and Western Europe (11–21%) [52]. In contradiction, the literature cites Coagulase-negative staphylococci as a characteristic complication of LOS [53]. Of the total of 21 infants with EOS, the one who died had the blood culture positive for Staphylococcus capitis. Interesting, this pathogen has been isolated in the intensive care units of seventeen countries. Given the increased drug resistance, the unfavorable prognosis was not surprising.

As both CRP and PCT are influenced by gestational age and birth weight, it is also important to take into consideration the optimal time of determination [54]. It was demonstrated that term infants have higher CRP than preterm [55]. Eschborn et al., reviewed the kinetics of PCT and CRP, and concluded that in order to rule out EOS, it is more conclusive to determine CRP and PCT at 12 hours of life rather than immediately after birth [56]. They also emphasized that serial determinations of these markers and their correlation with clinical findings were needed to support the decision of antibiotic therapy.

Neonatal complications (asphyxia, meconium aspiration, shock and intraventricular hemorrhage) and maternal risk factors (prolonged labor, PROM) may also cause increase values of CRP [57]. In this context, there has been a constant quest for other markers that could be included in the laboratory panels used for evaluation of newborns suspected of EOS. Newer studied markers like endocan, have elevated levels in newborns with sepsis (both early-onset and late-onset) and it is not influenced by gestational age, sex, fetal distress (meconium-stained amniotic fluid), delivery method or minor birth trauma [58–60].

According to recent studies, presepsin may be a better marker than CRP and PCT for the diagnosis of EOS and for monitoring the response to therapy. Its value rise early in the umbilical cord blood of the newborns with PPROM, does not vary with GA, postnatal age or with perinatal factors and decreases progressively with the administration of antibiotics [61,62]. However, these biomarkers were not available for evaluation in our cohort of patients.

Another study which investigated WBC, PLT and CRP in the diagnosis of EOS, reported normal values for WBC and PLT in both day 1 and day 3, but high values of CRP in both days, with an even elevated value in day 3 [41]. In our study, 7 (70%) patients with EOS (subgroup A1) showed high levels of CRP in day 1 and 3, while 6 (60%) of them had elevated CRP in day 5. The majority of neonates (n=8; 72%) with EOS and PROM <18 hours showed high CRP levels in day 3. Furthermore, the results from our random Effects GLS regression indicated as statistically significant predictors of neonatal sepsis were the white blood cells count from the first (p= 0.005) and third day (p= 0.028), and CRP values from the first day (p= 0.004).

A biomarker is considered good if the AUC is higher than 0.75 or excellent if greater than 0.9, respectively. Hence, in our study, out of all the researched biomarkers, procalcitonin (AUC - 0.78) and CRP measured on the first day (AUC - 0.76) had the best predictive performance for early onset neonatal sepsis. Even if we obtained good results, and procalcitonin is known as an early biomarker

for neonatal sepsis, there are studies which state that it may also be increased in healthy newborns, and could be more accurate in diagnosing late onset neonatal sepsis [63]. Moreover, our results showed that a combination of biomarkers (WBC, CRP, and fibrinogen), evaluated on the first and third day of life, had superior accuracy in detecting early onset neonatal sepsis (AUC: 0.83, AND 0.90, respectively).

Stocker et al., studied the relationship between simultaneous determination of CRP, PCT and WBC in no sepsis, sepsis uncertain, sepsis probable and sepsis proven patients [43]. When comparing the proven sepsis group with the no sepsis one, they reported an AUC of 0.986 for CRP, and an AUC of 0,921 for PCT, and those values increased with extended time frames up to 36 hours, whereas there was no difference between start to 36 hours vs start to 48 hours. In our study, although the AUC for CRP and PCT were lower, they did decrease between the first, second, and third determination. This change in the biomarker's dynamic could be the result of antibiotherapy.

This study has the following limitations: retrospective design, unbalanced data for the proven sepsis subgroups, limited number of biomarkers included. Further studies, on larger cohorts of neonates with early onset sepsis, that would include multiple panels of biomarkers could offer a more consistent perspective over the topic.

Supplementary Materials: Figure S1. AUC WBC day 1; Figure S2. AUC WBC day 3; Figure S3. AUC WBC day 5; Figure S4. AUC CRP day 1; Figure S5. AUC CRP day 3; Figure S6. AUC CRP day 5; Figure S7. AUC FIB day 1; Figure S8. AUC FIB day 3; Figure S9. AUC FIB day 5; Figure S10. AUC I/T; Figure S11. AUC PCT; Figure S12. AUC I/T+PCT; Figure S13. AUC parameters day 1; Figure S14. AUC parameters day 3; Figure S15. AUC parameters day 5.

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