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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

IMMUNOGENETIC CRITERIA OF NECROTIZING ENTEROCOLITIS

Specialty: 3220.01 – Pediatrics

Science field: Medicine

Applicant: Tarana Suleyman Humbatova

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The work was perfermed out at the Scientific Research Institute of Pediatrics named after K.Y. Farajova

Scientific supervisor: Honored Scientist,
Doctor of Medical Sciences,
Professor
Nasib Jafar Guliyev

Official opponents: Doctor of Medical Sciences,
Professor
Alakbar Gazanfar Hasanov

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Doctor of Medical Sciences
Huseynagha Ali Gurbanov

Doctor of Philolosophy of
Medical Sciences
Samaya Farzulla Alizade

Dissertation Council ED 2.27 of Supreme Attestation Commission under the President of the Republic of Azerbaijan operating at the Azerbaijan Medical University

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Vasadat Ali Azizov

Scientific secretary of the Dissertation Council: Doctor of Philolosophy of
Medical Sciences
Tora Akif Sadigova

Chairman of the Scientific Seminar: Honored Scientist,
Doctor of Medical Sciences,
Professor
Ibrahim Isa Isayev

GENERAL CHARACTERISTICS OF THE RESEARCH

Relevance of the topic. Necrotizing enterocolitis (NEC) is one of the most severe gastrointestinal diseases in newborns, especially in preterm infants. According to some researchers, this disease is a non-specific inflammatory condition caused by infectious agents against the background of immature local defense mechanisms. It leads to the generalization of inflammatory reactions due to hypoxic-ischemic damage to the intestinal mucosa and is considered a significant issue for newborns[1,2]. Infants who develop NEC are at a higher risk of infections compared to gestational age-matched infants. They absorb nutrients at a lower level, grow more slowly, require prolonged intensive care, and have longer hospital stays[3].

Worldwide, necrotizing enterocolitis develops in 6–15% of newborns[4]. Necrotizing enterocolitis affects from 2% to 5% of all preterm infants and is responsible for nearly 8% of neonatal intensive care unit (NICU) hospitalizations. In general, mortality ranges between 10% and 50%. However, in more severe cases accompanied by perforation, peritonitis, and sepsis, the fatality rate approaches 100%. Accurate early diagnosis and timely initiation of treatment are crucial[5].

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[1] Cuna A. Genetic predisposition to necrotizing enterocolitis in premature infants: Current knowledge, challenges, and future directions /A.Cuna, L.George, V.Sampath // Semin Fetal Neonatal Med., - 2018. Dec; 23(6), -p.387-393.

[2] Afzal B. Early onset necrotizing enterocolitis (NEC) in premature twins/ B.Afzal, V.Elberson, C.McLaughlin [et all.] // J Neonatal Perinatal Med., -2017. 10(1), -p.109-112.

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[4] Caplan M, Portman R. Second Annual Neonatal Scientific Workshop at the EMA Report. London: International Neonatal Consortium, -2016.

[5] Абдуманонов А. "Оптимизация лечения некротизирующего энтероколита у новорожденных" // -Ukraine: Science and innovation, -2022. Vol. 1, no. D8, -p.653-657. doi:10.5281/zenodo.7433165.

Despite certain successes in the diagnosis and treatment of NEC (Necrotizing Enterocolitis), there are no unified, specific, and sensitive methods to detect and predict the development of complications in newborns with early-stage NEC[6].

The diagnosis of early stages of the disease in low-birth-weight infants is extremely challenging. The active investigation of the most important diagnostic factors in the early stages of the disease is considered one of the primary tasks of neonatologists[7].

Despite the achievements made in the last decade, the pathophysiological aspects of NEC are still being clarified Thus, a highly active immune response is considered the primary provoking factor for many symptoms of NEC[8].

The aberrant activation of intestinal immune responses by the bacteria of the digestive system can cause inflammation and damage to the mucosal lining during NEC. In the immature intestine, the family of receptors that recognize pathogenic microorganisms (Toll-like receptors-TLRs) maintains a critical balance between bacterial tolerance and resistance[9].

Many genes with single nucleotide polymorphisms determine the risk and severity of NEC. Therefore, in modern times, genetic

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[6] Alsaied A. Global incidence of necrotizing enterocolitis: A systematic review and meta-analysis / A.Alsaied, N.Islam, L.Thalib // BMC Pediatr., -2020. 20:344. doi: 10.1186/s12887-020-02231-5.

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[8] Pang Y. Impairment of regulatory T cells in patients with neonatal necrotizing enterocolitis / Y. Pang, X. Du, X.Xu [et all.] // Int Immunopharmacol., -2018. Oct; 63, -p.19-25. doi:10.1016/j.intimp.2018.07.029.

[9] Sampath V. A functional ATG16L1 (T300A) variant is associated with necrotizing enterocolitis in premature infants / V.Sampath, V.Bhandari, J.Berger [et all.] // Pediatr Res., -2017. Ap; 81(4), -p.582-588. doi:10.1038/pr.2016.260. Epub 2016 Nov 28.

variations are being extensively studied. They have either been specifically identified during NEC or detected as similar histopathological deviations in other inflammatory bowel diseases[10].

In the context of a deeper study of NEC pathogenesis, scientific studies conducted at the molecular and cellular levels are the primary focus. Thus, in recent years, special importance has been given to the research of several informative laboratory indicators, inflammatory, and pro-inflammatory mediators in NEC pathogenesis. The conducted study has been specifically aimed in this direction.

Object and subject of the research. As part of the study, 80 newborns with necrotizing enterocolitis were examined (main group). Based on gestational age, the main group included 37 full-term and 43 preterm infants. The control group consisted of 17 practically healthy newborns without digestive system pathology, including 7 full-term and 10 preterm infants.

Purpose of the study: The aim was to study the clinical and immunogenetic criteria for the early diagnosis of NEC in newborns. In line with this objective, the following tasks have been set.

Tasks of the study:

1. Studying the characteristics of risk factors and clinical forms of necrotizing enterocolitis in newborns depending on gestational age (full-term and preterm birth).

2. Evaluating the serum levels of humoral immunity indicators (immunoglobulin A(IgA) and immunoglobulin G (IgG) affecting the course of necrotizing enterocolitis in newborns.

3. Evaluating levels of the pro-inflammatory and anti-inflammatory mediators (Platelet-activating factor (PAF), interleukin-8, (İL-8), interleukin-10 (İL-10)) affecting the course of necrotizing enterocolitis in the blood serum of newborns.

4. Studying the effect of IL-10 gene single nucleotide polymorphism on IL-10 cytokine secretion in newborns with necrotizing enterocolitis.

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[10] Donda KT. Single Nucleotide Polymorphisms in Neonatal Necrotizing Enterocolitis / KT.Donda, BA.Torres, M.Khashu [et all.] // Curr Pediatr Rev. - 2022. 18(3), -p.197-209. doi:10.2174/1573396318666220117091621

Research Methods.

Immunological examination methods

Genetic examination methods

Statistical examination methods

Main provisions submitted for defense:

-The antenatal risk factors differ depending on gestational age in patients with NEC.

-The study of cytokine status (PAF, IL-8, and IL-10) in patients with NEC has diagnostic significance.

-The single nucleotide polymorphism at the 1082 A/G position of the IL-10 cytokine gene affects the gene product.

Scientific novelty. For the first time:

- The characteristics of risk factors and clinical forms of necrotizing enterocolitis depending on gestational age have been studied.
- Changes in humoral and cellular immunity indicators (PAF, IL-8, IL-10, IgA, IgG) in the blood, depending on the clinical course of necrotizing enterocolitis in newborns, have been studied.
- The single nucleotide polymorphism at the 1082 A/G position of the IL-10 gene in newborn patients with necrotizing enterocolitis has been studied.

Practical significance of the research: The suitability and practical significance of the correct assessment of antenatal risk factors contributing to the development of necrotizing enterocolitis by neonatologists, the timely identification of newborns at risk of NEC through the study of cytokine status (levels of PAF, IL-8 and IL-10 in blood serum), and the use of their changing levels as a reliable criterion according to the severity of NEC have been proven. In subsequent research on NEC patients, the effectiveness of using the anti-inflammatory cytokine IL-10 and its recombinant gene product in reducing or preventing inflammatory reactions has been substantiated by genetic examination results.

Application of the research results. The results of the dissertation work have been applied in the clinical practice of the Scientific Research Institute of Pediatrics named after K.Y. Farajova.

Approbation. The results of the research were presented and discussed at the following conferences: The 26th International Neonatal Conference (UNEKO-2018) held in the Turkish Republic of Northern Cyprus in 2018, The All-Russian Scientific-Practical Conference with international participation held in Penza in 2018, the 62nd Turkish National Pediatrics Conference held in Antalya in 2018, the "Healthy Growing Children" conference organized by the Faculty of Health Sciences of Katip Çelebi University in Izmir in 2018, the XVIII International Conference on European-Asian Surgery and Hepatogastroenterology held in Baku in 2019, the conference dedicated to the 90th anniversary of Professor Demir Hajiyev at the Azerbaijan National Academy of Sciences in 2019, the TOBB ETÜ Pediatrics Symposium in 2020, the scientific-practical conference dedicated to the birthday of Aziz Aliyev at the Azerbaijan State Advanced Training Institute for Doctors named after Aziz Aliyev in 2020.

The preliminary discussion of the dissertation took place at a meeting of the Scientific Council of the Scientific Research Institute of Pediatrics named after K.Y. Farajova (Protocol №-1).

Personal involvement of the author in the research.

All stages of the research were personally carried out by the applicant.

Publications: Sixteen scientific works covering the results of the dissertation have been published, of which 6 are articles and 10 are theses.

Organization where the dissertation was conducted. Scientific Council of the Scientific Research Institute of Pediatrics named after K.Y. Farajova

The structure and volume of the dissertation

The dissertation consists of 157 computer pages and is illustrated with 14 figures and 31 tables. The total character count in the dissertation is 190.848 The dissertation work consists of Introduction (7354 characters), Literature Review (48,147characters), Materials and Methods (22,291 characters), Chapter III containing results and discussion (58,472 characters), Chapter IV (21,032 characters), Conclusions (30,736 characters),

Results (2028 characters), Practical Recommendations (788 characters), and References. The reference list comprises 152 sources, including 7 in Azerbaijani, 11 in Russian, and 134 in foreign languages.

MATERIALS AND METHODS

The research was conducted at the Scientific Research Institute of Pediatrics named after K.Y. Farajova between 2016 and 2018. The clinical part of the study was carried out in the departments of Resuscitation and Intensive Care, Pathology of Full-Term Newborns, and Early Childhood Surgery, while the laboratory part was conducted in the Scientific Diagnostic laboratory of the mentioned institute. A total of 80 newborns with necrotizing enterocolitis were examined (main group). Based on gestational age, the main group included 37 full-term and 43 preterm newborns. The control group consisted of 17 practically healthy newborns without gastrointestinal pathology, of whom 7 were full-term and 10 were preterm newborns.

Inclusion criteria for the main study group:

-Newborns with a primary clinical diagnosis of necrotizing enterocolitis confirmed by radiological methods;

-Patients diagnosed with necrotizing enterocolitis during the neonatal period who were admitted to the Intensive Care and Resuscitation department or the Neonatal Pathology department;

-Parental consent for participation in the study.

Exclusion criteria from the study:

- Newborns with congenital gastrointestinal malformations;
- Parental refusal to participate in the study.

Inclusion criteria for the control group:

- Infants born from pregnancies with a physiological course;
- Various gestational ages;
- Anthropometric indicators appropriate for gestational age at birth;
- An uncomplicated early neonatal period;
- Parental consent for participation in the study.

Exclusion criteria from the control group:

- Presence of perinatal pathologies;
- Parental refusal to participate in the study.

The clinical staging of necrotizing enterocolitis was conducted according to the Bell classification. Based on the Bell classification, patients in the main group were divided into the following categories:

Group I – 29 patients with NEC I (suspected NEC);

Group II – 26 patients with NEC II (definite NEC);

Group III – 25 patients with NEC III (progressive NEC).

All patients included in the examination underwent neurosonography, echocardiography, abdominal ultrasound, and X-ray imaging of the chest and abdomen. When it was necessary, contrast radiography (barium sulfate passage of the gastrointestinal tract, iridography) was also performed.

Laboratory Methods. Biochemical, serological, and bacteriological tests were conducted on the newborns included in the study. In the main group, general and bacteriological analyses of blood and stool, as well as blood gas content analysis, were performed in the clinical and bacteriological laboratories of the Scientific Research Institute of Pediatrics named after K.Y. Farajova.

Immunological examination methods.

Immunological examinations were conducted in the Immunological Laboratory section of the Clinical-Diagnostic Laboratory department of the Scientific Research Institute of Pediatrics using the immunoenzyme analysis method on the Elisys UNO – Human Fully Automated ELISA Analyzer. Blood samples (2 ml) were collected from peripheral veins of newborns with clinical signs of NEC into EDTA tubes on the day of observation (days 7-10 of life) and from newborns in the control group on the 3rd postnatal day. The samples were stored at -20°C and examined after all materials were ready, with the blood warmed to room temperature. A wavelength of 450 nm was selected as the primary filter for the measurements.

For the determination of cytokines in the peripheral blood of the examined newborns the follofing reagent sets were used: For the determination of PAF (Platelet-Activating Factor), the "Human

(PAF) ELISA Kit" (Sun Red Bio Biotech Co., Ltd, Shanghai Shanghong), for the determination of IgA, the "Human (IgA) ELISA Kit" (Sun Red Bio Biotech Co., Ltd, Shanghai Shanghong), for the determination of IgG, the "Human (IgG) ELISA Kit" (Sun Red Bio Biotech Co., Ltd, Shanghai Shanghong), for the determination of Interleukin-8 (IL-8), the "Human (IL-8) ELISA Kit" (Sun Red Bio Biotech Co., Ltd, Shanghai Shanghong), for the determination of Interleukin-10 (IL-10), the "IL-10 EASIA" (DIAsource, Louvain-la-Neuve, Belgium). The levels of cytokines were measured according to the protocols provided by the manufacturing companies. Humoral immunity markers Ig G and IgA were studied using serological tests.

Genetic examination methods. Single nucleotide polymorphism at position -1082 A/G of the interleukin-10 gene was studied by PCR-RFLP (Polymerase Chain Reaction Restriction Fragment Length Polymorphism) method at the Institute of Genetic Resources, MSE AR.

Statistical examination methods. Statistical processing of data was carried out using MS Excel -2010 software, the results were displayed in tables. The obtained indicators were arranged in order of variation and for each row, the mean value (M) and the mean error of this value (m) were calculated, the minimum (min) and maximum (max) values were shown.

In the initial stage of statistical analysis, parametric methods (Student's t-test) were applied. Subsequently, to refine the results, non-parametric methods were used, taking into account the number of indicators in the groups. The statistical significance of differences between groups was determined using Pearson's chi-squared (X^2) test, and differences were considered significant at $p < 0.05$. For processing quantitative indicators in the groups, non-parametric methods (ANOVA, Mann-Whitney U test, Univariate Analysis of Variance) were applied. To determine the correlation between two variables, Spearman's Rho test was used. Differences were considered statistically significant when the levels of the compared indicators were at $p < 0.05$.

The statistical analysis of polymorphic allele frequencies at the studied position of the IL-10 gene in NEC patients and the control group was performed using the SPSS computer program with the χ^2 (chi-squared) test.

RESULTS AND DISCUSSION

In the current research, we performed a comparative analysis of the indicators of full-term NEC patients with those of a control group consisting of healthy term infants with a gestational age of 38 weeks or more. According to the statistical analysis, the average birth weight in the control group was 3600 ± 81,5 g (min 3300 - max 4000 g), while in the NEC group, it was 2930 ± 102 g (min 1700 - max 4200 g). The average birth length in the control group was 51,4 ± 0,5 cm (min 50 - max 54 cm), while in the NEC group, it was 49,2 ± 0,4 cm (min 44 - max 56 cm). When comparing the indicators, a statistically significant difference was found between the NEC group and the control group in terms of birth weight and length ($p < 0,05$). Thus, full-term infants with NEC had lower birth weight and length compared to healthy newborns. We explained this result by the complicated obstetric history of the mothers of full-term infants who developed NEC. In the subsequent stage of the study, we investigated the obstetric-gynecological and pregnancy complications of the mothers of full-term healthy infants and those with NEC included in the examination. Based on the results, contrary to the control group, 89,2% of the mothers of full-term newborns with NEC had anemia, 81,1% had preeclampsia, 43,2% had chorioamnionitis, 37,8% had somatic diseases, 24,3% had nephropathy, and among the TORCH (toxoplasmosis, rubella, cytomegalovirus, and herpes) infections, 70,3% had toxoplasmosis, 26% had cytomegalovirus, and 19,7% had herpes. Especially, in mothers of full-term infants with NEC, the frequency of preeclampsia, anemia, and toxoplasmosis was significantly higher compared to mothers of full-term healthy infants ($p < 0,001$). Additionally, the frequency of maternal somatic diseases and

cytomegalovirus infection showed statistically significant differences ($p < 0,05$).

It is known that during pregnancy, the mentioned pathologies in mothers—preeclampsia (systolic blood pressure exceeding 140 mm Hg and proteinuria after the 20th week of pregnancy), severe anemia (Hct <30%), intrauterine infections, and chorioamnionitis (antenatal pre-inflammatory stage involving the placenta and fetal membranes)—lead to disturbances in placental function, a decrease in maternal-placental-fetal circulation, i.e., reduced fetal blood flow, and ultimately result in chronic fetal hypoxia and intrauterine growth restriction. At the early stages of pregnancy, disturbances in placental function can be compensated by the high adaptability of the maternal-placental-fetal circulation, ensuring the fetus's viability. However, with the increasing effects of pathological factors and prolonged exposure, deepening hypoxia leads to increased resistance in the uterine spiral arteries and later in the peripheral vessels of the fetal part of the placenta. This results in decreased perfusion of the placenta and reduced blood flow in the umbilical vein. At this stage, blood centralization in the fetus is compensatory in nature. Thus, blood flow accelerates to vital organs such as the brain, myocardium, and adrenal glands. The amount of blood supplied to the abdominal organs, including the intestines, decreases. This ultimately leads to hypoxic-ischemic damage in these areas. It is not coincidental that in our research when examining the ante-intra and postnatal clinical characteristics of the study group, i.e., full-term newborns with NEC, the main perinatal risk factors for NEC were found to be perinatal hypoxic-ischemic encephalopathy (59,5%) and intrauterine growth restriction (45,9%), both of which developed against the backdrop of increasing hypoxia. It was detected that both of these pathological processes occurred in mothers with severe anemia (89,2%), preeclampsia (81,1%), and toxoplasmosis infection (70,3%). Thus, a strong correlation was found between the complicated pregnancy pathologies in mothers and the mentioned early neonatal complications in newborns, with a high correlation coefficient ($r=0.8$, $p<0,001$). In full-term NEC patients, the other perinatal complications we observed more frequently were birth asphyxia

(40,5%), respiratory therapy (35,1%), and patent ductus arteriosus (37,8%) and significant differences in these indicators were observed compared to the healthy group ($p<0,05$).

In the current research, full-term NEC patients were also infants born with low Apgar scores. Their 1-minute Apgar score (6 ± 0,3, min 0 - max 8) was lower compared to the healthy group (7,9 ± 0,1, min 7 - max 8) ($p < 0,05$), and their 5-minute Apgar score (7,1 ± 0,2, min 3 - max 9) was more significantly lower, showing a highly significant difference compared to the healthy group (8,7 ± 0,2, min 8 - max 9) ($p < 0,001$). In newborns who experienced birth asphyxia and were assessed with low Apgar scores at birth, the diversion of blood to vital organs results in the intestinal mucosa remaining hypoxic in the postnatal period.

When examining the clinical features of the NEC patients included in the study group, among full-term patients, the most commonly observed signs were abdominal distension (94,6%), bilious gastric contents (91,9%), vomiting (73%), painful abdomen on palpation (51,4%), blood in the stool (51,4%), and diminished bowel sounds on auscultation (40,5%). A noteworthy finding in our study was that the clinical signs of NEC appeared earlier, within the first 8–9 days of life, in full-term infants. It is known that the clinical manifestations of NEC usually develop within the first 14 days after birth, mainly following the initiation of enteral feeding. However, as we mentioned, in our study, we did not observe a relationship between the clinical signs of NEC and enteral feeding in full-term patients and clinical symptoms were detected within the first 8–9 days of life. We attribute the early onset of clinical manifestations to the earlier occurrence of hypoxic-ischemic processes in the intestinal tissue of the patients in our study group, beginning during the intrauterine and intranatal periods.

In our study, preterm NEC patients had a body weight of 1926±64,3 g (min 1000 – max 2300) and a body length of 44,4±0.3 cm (min 40 – max 43). Compared to the control group (2325±96,5 g (min 1800 – max 2670) and 46.6±0,5 cm (min 43 – max 49)), a statistically significant difference was found in anthropometric indicators ($p<0,05$). In other words, similar to term infants, preterm

infants also developed NEC, and their body weight and length were lower compared to the control group. Numerous clinical and demographic studies aimed at identifying risk factors for NEC have also specifically highlighted preterm birth and low birth weight as significant risk factors for the development of NEC.

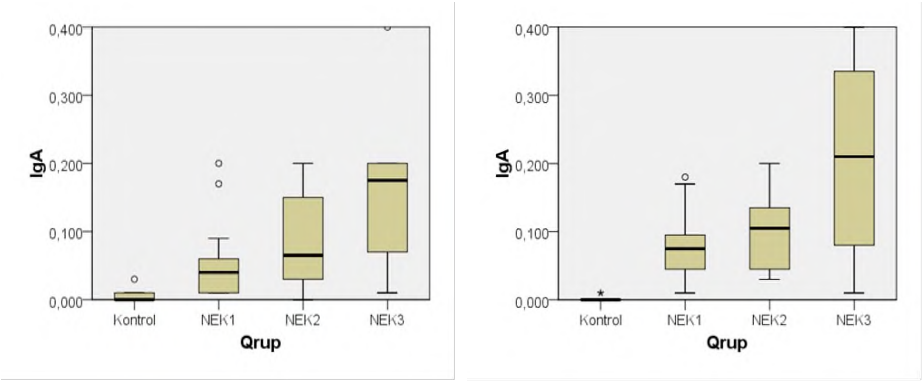
An interesting finding in the study was that mothers of preterm infants who developed NEC had a higher likelihood of experiencing intrauterine infections compared to mothers in the control group. In our current study, 65,1% of preterm NEC patients were found to have cytomegalovirus infection, while 60,5% had toxoplasmosis infection. A statistically significant difference was observed in these indicators compared to the control group ($p<0,05$). This suggests that intrauterine infections occurring during pregnancy, which can cause significant functional changes in various fetal organs and systems, may contribute to the development of NEC in the neonatal period, especially in preterm infants.

In the current study, the most frequently observed concomitant diseases in preterm NEC patients were anemia (67,4%) and perinatal hypoxic-ischemic encephalopathy (65,1%), with a statistically significant difference ($p<0,001$). The incidence rates of birth asphyxia (51,2%), respiratory distress syndrome (46,5%), sepsis (34,9%), and erythrocyte mass transfusion (30,2%) showed statistically significant differences ($p<0,05$). Furthermore, 37,2% of NEK patients with concomitant respiratory distress syndrome received surfactant therapy.

As the clinical course of the disease worsens, both cellular and humoral immunity factors play a role in regulating these laboratory markers, which characterize tissue damage and inflammatory processes in the general blood analysis. Humoral immunity is considered phylogenetically older and assumes the primary defense function until more advanced immune mechanisms develop. This contributes to the relative resistance of neonates to certain infectious diseases. In our study, we identified changes in the levels of cytokines—both pro-inflammatory (PAF, IL-8) and anti-inflammatory (IL-10)—as well as immunoglobulins, which are factors of cellular immunity, in NEC patients. These changes were

observed not only when comparing NEC-affected neonates with the healthy group but also across different clinical stages of NEC (NEC I, NEC II, and NEC III).

Thus, in our current study, we observed a significant increase in IgA levels in the full-term NEC group (0,085±0.014 mg/ml (min 0,00 – max 0,4)) compared to the healthy group (0,007±0,004 mg/ml (min 0,00 – max 0,03)), with a statistically significant difference ($p<0,05$). In the intergroup comparison, we identified a significant difference in IgA levels among full-term NEC patients ($p < 0,05$). In the NEC III group (0,172 ± 0,055 mg/ml, min 0,01 - max 0,4), the IgA level was more than twice as high as in the NEC I group (0,053 ± 0,014 mg/ml, min 0,01 - max 0,2), which was particularly noteworthy (Graph 1a).

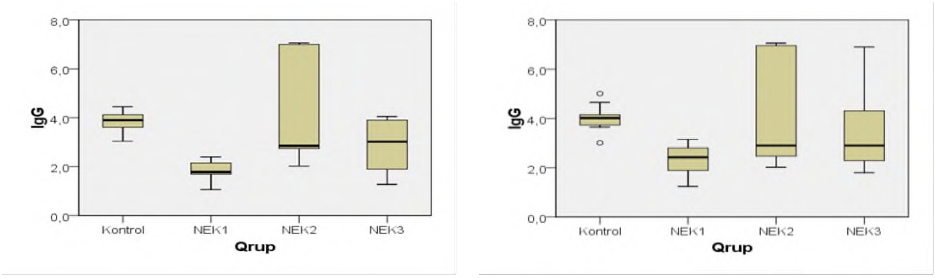


a) **b)**

Graph 1. The average IgA level in the blood of term (a) and preterm (b) neonates in the control and NEC groups (mg/ml)

In the preterm NEC patient group, the average IgA level in plasma was 0,143 ± 0,018 mg/ml (min 0.01 - max 0.4), while in the control group, it was 0,002 ± 0.001 mg/ml (min 0,00 - max 0.01). The results showed a highly significant difference between the groups ($p < 0,001$). When comparing IgA levels among the groups organized according to the severity of NEC, a significant difference was found ($p < 0,05$) (Graph 1b).

When examining IgG levels, no significant difference was found between the full-term NEC group (2,91 ± 0,29 mg/ml, min 1,06 - max 7,06) and the healthy group (3,84 ± 0,18 mg/ml, min 3,04 - max 4,45) (p > 0,05). However, in the intergroup comparison, the IgG level in the NEC II group (3,20 ± 0,58 mg/ml, min 2,02 - max 7,06) was significantly higher than in the NEC I group (1,87 ± 0,08 mg/ml, min 11,06 - max 2,4). In contrast, the IgG level in the NEC III group (3,86 ± 0,45 mg/ml, min 1,27 - max 4,05) was significantly lower compared to the NEC II group (3,20 ± 0,58 mg/ml, min 2,02 - max 7,06), which was particularly notable (Graph 2a).



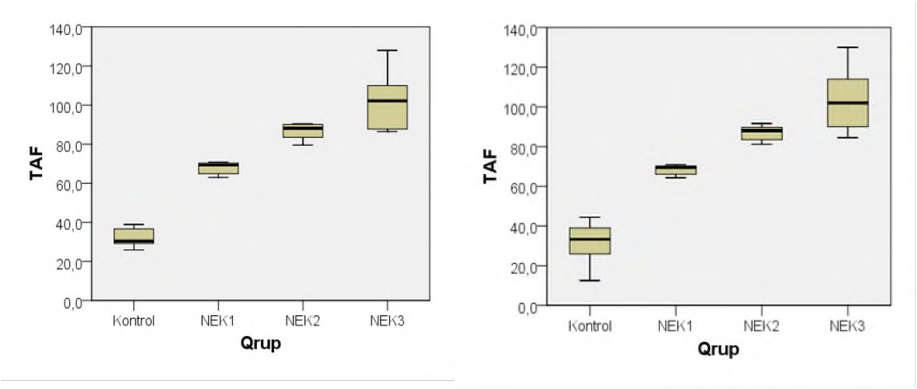
a) **b)**

Graph 2. The average IgG level in the blood of term (a) and preterm (b) neonates in the control and NEC groups (mg/ml)

In the preterm NEC patient group, the average IgG level in blood plasma was found to be 3,24±0,24 mg/ml (min 1,24 – max 7,06), while in the control group, it was 4,02±0.17 mg/ml (min 3,01 – max 5,01), and no statistically significant difference was observed (p>0,05). When comparing IgG levels in groups based on the severity of NEC, the levels were as follows: NEC I 2.33±0,17 mg/ml (min 1,24 – max 3,15) in the group, NEC II 3,06±0,64 mg/ml (min 2,02 – max 7,06) in the NEC II group and NEC III 3,31±0,31 mg/ml (min 1,80 – max 6,90) in the group. In preterm NEC patients, as the clinical course worsened, a statistically significant difference in IgG levels was detected (p>0,05) (Graph 2b).

In modern research on the pathogenesis of NEC, PAF has begun to be recognized as a prospective biomarker. Studies

conducted on animal models have shown that PAF stimulation via lipopolysaccharides activates the immune signaling of cells, leading to abnormal expression of TLR4. Additionally, a parallel relationship has been observed between the increased levels of PAF and ischemic damage in the intestinal tissue, as demonstrated histologically. However, there are few scientific studies investigating the levels of PAF in the blood during NEC in neonates. In our current study, we performed a comparative analysis of PAF levels in the blood plasma of term infants with NEC and healthy neonates. Based on our findings, the average levels of PAF in the blood were as follows: 32,4 ± 1.9 ng/ml (min 25,9 – max 38,9) in the control group, 68,0 ± 0,7 ng/ml (min 63 – max 70,7) in the NEC I group. Based on clinical course, this parameter was found to be 86,5 ± 1.1 ng/ml (min 79,5 – max 90,5) in the NEC II group, and 102,8 ± 6,5 ng/ml (min 86,5 – max 128) in the NEC III group (Graph 3a).



a) **b)**

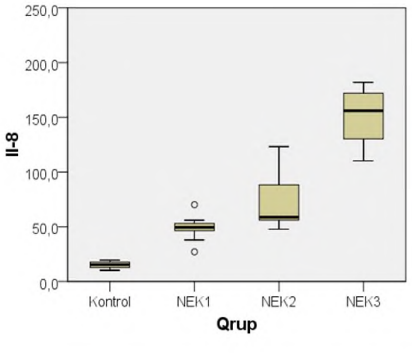
Graph 3. The average PAF level in the blood of term (a) and preterm (b) neonates in the control and NEC groups (ng/ml)

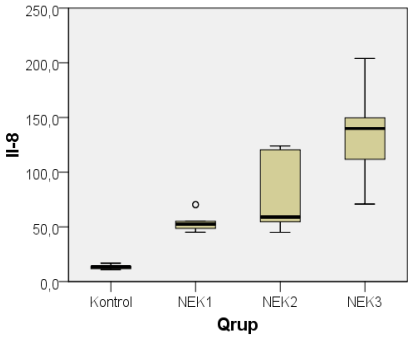
In preterm infants, the average levels of PAF in the blood were as follows: 89,3 ± 2,8 ng/ml (min 64,3 – max 130) in the NEC group and 31,4 ± 3,1 ng/ml (min 12,5 – max 44,4) in the control group. Based on clinical course, this parameter was found to be 68,4 ± 0,7 ng/ml (min 64,3 – max 70,9) in the NEC I group, 86,7 ± 1,1

ng/ml (min 81,2 – max 91,6) in the NEC II group, and 104 ± 3,5 ng/ml (min 84,5 – max 130) in the NEC III group. The results obtained showed a statistically significant difference between both term and preterm NEC patients compared to the control group, as well as among the clinical forms of NEC in the groups we formed ($p < 0,001$) (Graph 3b).

Thus, as in term NEC patients, PAF is also a promising biomarker for early diagnosis and clinical course assessment in preterm NEC patients. A notable finding in our results is that the average plasma PAF level in term NEC patients (80,6 (63–128) ng/ml) was recorded as higher compared to the average plasma PAF level in preterm NEC patients (89,3 (64,3–130) ng/ml). We attribute this result to findings from studies on preterm neonates, which indicate that PAF-AH enzyme levels are lower in preterm infants compared to term infants and after 36 weeks of gestation, PAF-AH enzyme in blood plasma reaches normal levels.

We also analyzed IL-8, another pro-inflammatory marker. According to our findings, the IL-8 level in the term NEC patient group was 74,5 ± 6,8 pg/ml (min 27,1 – max 182), which was significantly higher compared to the control group (15,1 ± 1,4 pg/ml, min 10,2 – max 19,5) ($p < 0,001$). These results align with the findings reported in the literature. Within the NEC groups, IL-8 levels varied according to the clinical course of the disease. As the clinical severity of NEC increased, the IL-8 concentration in blood plasma also increased significantly ($p < 0,001$). The highest IL-8 concentration was observed in the NEC III group. Thus, the IL-8 level was found to be 151,1 ± 11,4 pg/ml (min 110,3 – max 182) in the NEC III group, 72,4 ± 7,5 pg/ml (min 47,8 – max 123,2) in the NEC II group, and 49,1 ± 2,1 pg/ml (min 27,1 – max 70,2) in the NEC I group ($p < 0,001$) (Graph 4a).





a) **b)**

Graph 4. The average IL-8 level (pg/ml) in the blood of term (a) and preterm (b) neonates

According to our results, in preterm neonates, the IL-8 level in the NEC group (96,6 ± 7,2 pg/ml, min 45,1 – max 204) was significantly higher compared to the control group (13,4 ± 0,6 pg/ml, min 11 – max 16,8) ($p < 0,001$) (Graph 4b).

An interesting aspect of our results is that the mean plasma IL-8 level in full-term healthy newborns (15,1 pg/ml) was higher than in the preterm control group newborns (13,4 pg/ml). We attribute this finding to the mode of delivery of the newborns included in the control group. Literature suggests that newborns delivered naturally have higher IL-8 levels in blood plasma compared to those born via cesarean section, which is associated with birth stress. 80% of the full-term newborns in our research were delivered naturally. Among the preterm births, 20% were delivered naturally, while 80% were delivered via cesarean section. Therefore, in the control group of our study, the percentage of natural births among full-term newborns was higher, and consequently, higher levels of IL-8 in plasma were detected in these infants. According to our results, the IL-8 levels in preterm neonates with NEC changed according to the clinical progression. As the clinical course of NEC worsened, the concentration of IL-8 in blood plasma significantly increased ($p<0,05$), with the highest concentration found in the NEC III group. The IL-8 levels were 135,7±8,8 pg/ml (min 70,9, max 204) in the NEC III group, 77,1±9,7 pg/ml (min 45,1, max 124) in the NEC II group, and 54,3±2,3 pg/ml (min 45,2, max 70,5) in the NEC I group.

The results obtained indicate that the increasing concentration of IL-8 in blood plasma has significant potential as a diagnostic marker for both term and preterm neonates with NEC. It could be an

important mediator in assessing the severity and prognosis of NEC. This cytokine's active form activates neutrophils, facilitating their movement, particularly their directed movement (chemotaxis). Several studies have shown that elevated levels of IL-8 in plasma are a significant indicator for the early diagnosis of bacterial infections in neonates. Lodha and co-authors analyzed the levels of PAF, IL-6, and IL-8 in the blood plasma of NEC patients. They also found a significant correlation between the levels of these cytokines in plasma and the future physical and psychomotor development of the patients. Dembinski and co-authors, on the other hand, studied the levels of IL-8 in the blood taken from the umbilical cord in both term and preterm neonates. However, there are no medical literature sources on studies examining the levels of IL-8 in the plasma of NEC patients based on gestational age and the severity of NEC. On the other hand, we also studied and analyzed the levels of IL-8 in the plasma of NEC patients along with the levels of PAF. In the comparison between groups based on the severity of NEC in both term and preterm NEC patients, the levels of PAF and IL-8 were significantly different ($r=0,698$, $p<0,001$). A strong positive correlation was observed between the increasing levels of these markers in the blood. The parallel increase in the levels of PAF and IL-8 characterizes the severity of NEC. Therefore, for early diagnosis, that is, to identify NEC patients and simultaneously assess the clinical course in these patients, PAF and IL-8 are prospective biomarkers.

The timely initiation of treatment and early diagnosis are crucial for the outcome of NEC. The use of antagonists for these mediators in the future (PAF antagonists, such as CV-3988 and SM-12502, apafant, modipafant, and the IL-8 antagonist actinomycin D) could be beneficial for modulating the initial production of PAF and IL-8 in an appropriate manner, potentially preventing the development of NEC.

The change in the laboratory results of PAF and IL-8 in NEC patients compared to the healthy group, as well as their variation depending on the clinical form and severity of the disease, sparked significant interest in our study. For this reason, we considered it

appropriate to investigate the levels of the anti-inflammatory cytokine IL-10 in plasma in relation to the clinical progression of NEC in our research.

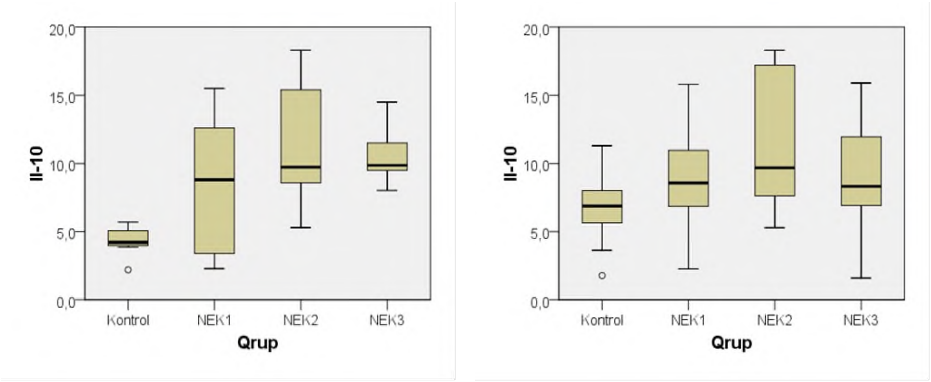
Numerous scientific studies have shown that IL-10 plays a protective role as an anti-inflammatory cytokine in intestinal tissue, and its deficiency has been found to further increase the degree of inflammation in tissues. Other research has indicated that pro-inflammatory cytokines such as PAF-α and IL-6 play a significant role in the pathogenesis of NEC, while among the anti-inflammatory cytokines, IL-10 may have a potential protective role. In experimental studies, targeted deletion of the IL-10 gene in animal models has resulted in the development of NEC. IL-10 deficiency significantly increased the severity of inflammation in the intestine, leading to an increased mortality rate, enterocyte apoptosis, and the development of macroscopic and microscopic tissue changes corresponding to the severity of NEC. Exogenous administration of IL-10 has been shown to restore those morphological changes. However, there are no studies on humans examining the protective role of IL-10 in patients with NEC. The research we conducted is the first study to investigate the protective role of the IL-10 cytokine in the pathogenesis of NEC and to examine the single nucleotide polymorphism of the IL-10 gene.

Based on our results, a significant increase in IL-10 levels was observed in term neonates with NEC compared to the control group ($p<0.05$). Thus, in neonates with NEC, the IL-10 level was 9,71±0,72 pg/ml (min 2,29, max 18,30), while in the control group, it was 4,31±0,43 pg/ml (min 2,20, max 5,70). When comparing IL-10 levels between groups, in the NEC I group, the IL-10 level was 8,34±1,13 pg/ml (min 2,29, max 15,5), in the NEC II group, 9,03±1,2 pg/ml (min 5,3, max 18,3), and in the NEC III group, 9,54±0,91 pg/ml (min 8,02, max 14,5) (Graph 5a).

No significant difference in IL-10 levels was observed between the groups ($p>0,05$). However, the higher levels of IL-10 in the II group compared to the control group and the higher levels in the II group compared to the I group suggest the systemic involvement of immune responses in the pathogenesis of NEC.

In preterm neonates with NEC, the IL-10 level was 9,65±0,64 pg/ml (min 1,6, max 18,3), while in the control group, it was 6,58±0,82 pg/ml (min 1,78, max 11,3).

On the other hand, our results showed that the average IL-10 levels in the plasma of term neonates in the control group were lower compared to those in the control group of preterm neonates. Thus, the average IL-10 level in the control group of term neonates was 4,31 pg/ml, while in the control group of preterm neonates, the average value was 6,58 pg/ml, which aligns with the results reported in the l



a) **b)**

Graph 5. The average IL-10 level (pg/ml) in the blood of term (a) and preterm (b) neonates

In a study conducted by Blanco-Quiros and colleagues, the IL-10 levels in plasma were measured in the umbilical cord blood of both term and preterm neonates. It was found that the levels of IL-10 were significantly higher in preterm infants compared to term infants. They linked this result to the immaturity of preterm neonates and the immunosuppression that occurs during pregnancy. It was found that the increase in IL-10 levels in these infants was inversely proportional to the gestational age, with the highest levels of IL-10

being recorded in neonates with the lowest gestational age. In other words, our results are consistent with the literature sources. When comparing the IL-10 levels between groups of preterm neonates with NEC, it was found that in the NECI group, the IL-10 level was 8,91±1,01 pg/ml (min 2,27, max 15,8), in the NECII group, it was 10,33±1,44 pg/ml (min 5,29, max 18,3), and in the NECIII group, it was 9,05±0,91 pg/ml (min 1,6, max 15,9). No significant difference in IL-10 levels was observed between the I, II, and III groups in preterm neonates with NEC (p>0,05) (Figure 5b).

In the conducted research, we also investigated the IL-10 cytokine from an immunogenetic perspective (Graph 6).

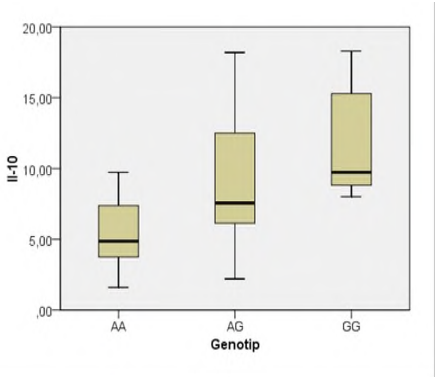


Figure 6. The effect of the genotype detected at position 1082 of the IL-10 gene on IL-10 levels in healthy and NEC-affected newborns

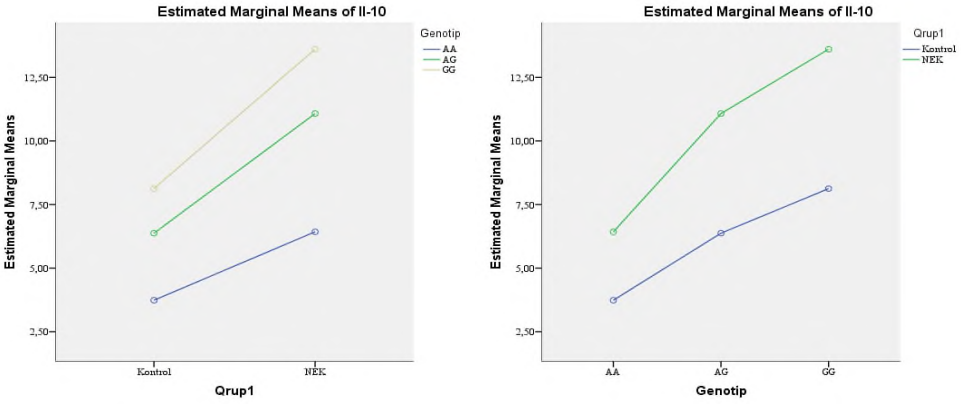
Our results showed that the substitution of adenine with guanine at position 1082 of the investigated promoter region of the IL-10 gene led to an increase in IL-10 protein levels in NEC-affected neonates. The IL-10 level was found to be the lowest in homozygous individuals with the AA genotype at 5,35±0,69 pg/ml (min 1,6 -max 9,73), intermediate in heterozygous individuals with the AG genotype at 9,38±0,86 pg/ml (2,2-18,2), and the highest in homozygous individuals with the GG genotype at 12,04±1,57 pg/ml

(8,01 – 18,3). The results showed a statistically significant difference ($p<0,05$).

In the statistical analysis conducted considering both the diseased and healthy groups, IL-10 levels were found to be low in homozygous individuals carrying the AA allele and high in homozygous individuals carrying the GG allele in both the NEC-affected and healthy groups (Graph 7)

The known gene polymorphism of the anti-inflammatory cytokine IL-10 has led to a decrease in its expression and an increase in the levels of inflammatory mediators.

Similarly, in the statistical analysis conducted based on genotype, an increase in IL-10 levels was observed from homozygous individuals carrying the AA allele to those carrying the GG allele in both the NEC-affected and healthy groups (Graph 7).



Graph 7. Results of two-factor analysis (uANOVA) for the IL-10 gene at position 1082, considering the diseased and healthy groups

Thus, it has been found that the body's response depends on the balance between an excessive inflammatory reaction and the counteracting anti-inflammatory response. In the pathogenesis of NEC, the progression and severity of the disease are also influenced by this balance, indicating that the regulation of the inflammatory

process in neonates differs. Normally, when the levels of pro-inflammatory cytokines reach a certain threshold, anti-inflammatory cytokines are activated, leading the inflammatory process toward recovery. In neonates, the regulation of the inflammatory process is disrupted due to immune immaturity. Besides, in some individuals with genetic differences, the increase in pro-inflammatory cytokines is not met with a corresponding rise in anti-inflammatory cytokines, ultimately leading to the development of severe inflammatory processes. From this perspective, further large-scale studies are needed to investigate the balance between pro-inflammatory and anti-inflammatory cytokines in NEC patients.

CONCLUSIONS

1. In term NEC patients, maternal conditions such as anemia (89,2%), preeclampsia (81,1%), and toxoplasma infection (70,3%) were significantly higher compared to the control group ($p<0,001$). In preterm NEC patients, maternal cytomegalovirus infection (65,1%), toxoplasma infection (60,5%), maternal somatic diseases (41,9%), and chorioamnionitis (39,5%) showed statistically significant differences compared to the control group ($p<0,05$) [9].
2. In term neonates with NEC, perinatal hypoxic-ischemic encephalopathy (59,5%), intrauterine growth restriction (45.9%), and birth asphyxia (40,5%) were observed ($p<0,05$). In preterm neonates with NEC, anemia (67,4%), perinatal hypoxic-ischemic encephalopathy (65,1%), birth asphyxia (51,2%), Respiratory Distress Syndrome (RDS) (46,5%), and sepsis (34,9%) were noted ($p<0,05$). In the preterm NEC group, clinical symptoms were observed at the end of the early neonatal period [14].
3. In all NEC patient groups, IgA levels in blood were elevated and showed a statistically significant difference compared to

the control group ($p<0,05$). In term NEC patients, intergroup comparison based on NEC severity revealed a highly significant increase in IgG levels ($p<0,001$). Similarly, in preterm NEC patients, intergroup comparison based on NEC severity showed statistically significant differences in IgG levels ($p<0,05$) [10].

4. In all NEC patient groups, levels of PAF, IL-8, and IL-10 in blood were elevated. Compared to the control group, PAF and IL-8 levels showed a highly significant difference ($p<0,001$), while IL-10 levels demonstrated a significant difference ($p<0,05$). In the comparison between groups of NEC (necrotizing enterocolitis) patients, both term and preterm, classified according to the severity of NEC, the levels of PAF and IL-8 were significantly higher and showed a strong positive correlation ($r = 0,698$, $p < 0,001$). The increasing levels of these markers in the blood were strongly and directly correlated. The parallel rise in PAF and IL-8 levels characterizes the severity of NEC[2,3,5,6,8,12] .

5. The substitution of adenine (A) with guanine (G) at the -1082A/G promoter region of the IL-10 gene led to an increase in the amount of IL-10 protein in both the control group and the NEC patient group. However, in NEC patients, the level of IL-10 in blood serum was lower in individuals carrying the AA allele compared to those carrying the GG allele [11,13,16].

PRACTICAL RECOMMENDATIONS

1. To reduce the probability of NEC occurrence, it is recommended to timely identify antenatal risk factors and implement prophylactic and therapeutic measures for NEC in newborns who have experienced perinatal hypoxic-ischemic encephalopathy, intrauterine growth restriction, birth asphyxia, anemia, respiratory distress syndrome ,and sepsis, and apply proper nutritional strategies, particularly breastfeeding.
2. It is recommended to study the cytokine status—specifically the levels of PAF, IL-8, and IL-10—in newborns with NEC and evaluate their increasing levels as a diagnostic criterion based on the severity of NEC.
3. Since the IL-10 cytokine plays a significant role in the pathogenesis of necrotizing enterocolitis, the lower level of IL-10 in blood serum observed in individuals carrying the AA allele is recommended as a diagnostic criterion for NEC.

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ABBREVIATIONS

IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-8	Interleukin -8
IL-10	Interleukin -10
NEC	Necrotizing Enterocolitis
RDS	Respiratory Distress Syndrome
PCR RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PAF	Platelet-activating factor
TLR	Toll-Like Receptor

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