C5a, A Complement Activation Product, Is a Useful Marker in Predicting the Severity of Necrotizing Enterocolitis

Cuneyt Tayman,1 Alparslan Tonbul,1 Hasan Kahveci,2 Sema Uysal,3 Burhan Koseoğlu,4 M. Mansur Tatlı1 and Ugur Dilmen5

1Department of Neonatology, Fatih University Faculty of Medicine, Ankara, Turkey
2Department of Neonatology, Nenehatun Women Health Hospital, Erzurum, Turkey
3Department of Biochemistry, Fatih University Faculty of Medicine, Ankara, Turkey
4Department of Pediatric Surgery, Fatih University Faculty of Medicine, Ankara, Turkey
5Department of Neonatology, Zekai Tahir Burak Maternal Health Teaching Hospital, Ankara, Turkey

Necrotizing enterocolitis (NEC) is the most common neonatal gastrointestinal emergency, predominantly affecting low-birth weight, premature infants. Ninety percent of cases occur in preterm infants, with the greatest risk in the smaller, more premature infants (Lin et al. 2008; Thompson and Bizzarro 2008; Schnabl et al. 2008). Compared with an overall incidence rate of 1% to 7.7%, up to 7% to 14% of very-low-birth-weight infants (< 1,500 g) are diagnosed with NEC (Kosloske 1994; Neu 2005). NEC is characterized by disruption of mucosal integrity, feeding intolerance, bloody stools, cardiorespiratory compromise, and severe hemodynamic instability. The etiology and pathophysiology of NEC is not fully understood, in spite of extensive studies (Lin et al. 2008; Thompson and Bizzarro 2008; Schnabl et al. 2008). NEC is a devastating disease with a mortality rate of 10% to 50% (Kosloske 1994; Thompson and Bizzarro 2008; Schnabl et al. 2008). Morbid sequelae among survivors include impaired growth, short bowel syndrome, prolonged neonatal hospitalization, and poor long-term neurodevelopment (Carter 2007; Schulzke et al. 2007). Despite advances in neonatal medicine, diagnosis of NEC remains a major challenge. Early clinical signs are nonspecific and the laboratory findings are not fully reliable. Therefore, its delayed occurrence after birth, its rapid onset, highly fulminant nature, and progression to death, as well as its severe morbidity, require identification of prospective new biomarkers specific for high NEC risk (Young et al. 2009). Some studies have suggested the efficacy of several inflammatory mediators such as C-reactive protein (CRP), interleukin-6 (IL-6) and serum amyloid A (SAA) measurements in the diagnosis of NEC (Pourcyrous et al. 2005; Hallstrom et al. 2006; Markel et al. 2006; Evennett et al. 2009; Cetinkaya et al. 2010). These all have difficulties that limit their use. Therefore, the application of new biomarkers would offer opportunities for early diagnosis and intervention in the disease.

The complement activation product, C5a, is a strong chemoattractant peptide involving the recruitment of
inflammatory cells such as neutrophils, eosinophils, monocytes, and T lymphocytes, and it also activates phagocytic cells to release granule-based enzymes and generate oxidants that contribute to innate immune functions or tissue damage (Kühl 2001; Guo and Ward 2005). The elevated levels of C5a have been shown to be increased in various diseases including pneumothorax, intracerebral hemorrhages, respiratory distress (Enskog et al. 1996) and early onset of infection in preterm infants which may be useful for diagnosis and follow-up (Zilow et al. 1997). In addition, C5a was reported to be a contributing factor leading to mesenteric ischemia/reperfusion injury (Fleming et al. 2003), which is a predisposing factor in the pathogenesis of NEC (Lin et al. 2008; Thompson and Bizzarro 2008; Schnabl et al. 2008). To our knowledge, levels of C5a have never been evaluated during NEC episodes in premature infants. Therefore, we aimed to evaluate the efficacy of serial C5a measurements in the diagnosis and follow-up of NEC, and to compare its effectiveness with CRP, IL-6 and SAA in NEC.

**Material and Methods**

The study was performed at two different tertiary referral centers for obstetrics and neonatal intensive care units from December 1, 2009 to May 1, 2010. Infants that were at ≤32 weeks of gestation and ≤1,500 g of birth weight were enrolled in the study. Ethics Committees at Fatih University Faculty of Medicine and Zekai Tahir Burak Maternal Health Teaching Hospital (Ankara, Turkey) approved the study with human subjects or materials, and informed consents were obtained from the parents. Gestational age was determined by clinical data and by a first trimester ultrasound scan. Maternal age and medical history, and the data for antenatal steroid treatment were obtained from the obstetrics and gynecology department records. Modes of delivery [by vaginal (NVD) or cesarean section (C/S)], gender, birth weight, APGAR scores at the 1st and 5th minutes and resuscitation requirements were recorded. Mechanical ventilation therapy, exchange transfusions, neural tube defects and congenital heart diseases were recorded before the diagnosis of NEC.

Clinical symptoms, such as increased episodes of apnea and desaturation, bradycardia, lethargy, irregular body temperature, feeding intolerance, vomiting, and findings such as increased gastric residual volume (>20%), bilious or bloody gastric aspirate, decreased bowel sounds, bloody stools, abdominal distention and tenderness, and abdominal wall skin color changes were evaluated. In infants with two or more of these symptoms/findings (these early gastrointestinal symptoms were common in patients with NEC), laboratory and radiographic evaluations were performed. Abnormal findings on abdominal radiographs including intestinal dilatation, the presence of dilated and fixed bowel loops, thickened intestinal wall, ascites, pneumatosis intestinalis, air in the portal vein, pneumoperitoneum and free air after perforation were evaluated by a radiologist. Laboratory studies, including, complete blood count, blood smear, levels of IL-6 and CRP, serum electrolytes, blood urea nitrogen, creatinine, liver function tests, urinalysis and blood cultures were evaluated. After diagnosis of NEC according to clinical and radiological findings, modified Bell criteria were used for staging of the disease as stage I, II or III (Walsh and Kliegman 1986). According to this staging system, stage I (suspicious) NEC was not taken into account, and only patients with true NEC at stage II (mild or moderate NEC) and stage III (advanced NEC) were allocated. The patients were followed by a single team of specialists including pediatric surgeons and neonatologists. The same team made the decision for surgical intervention, if necessary. Findings of pneumoperitoneum and/or necrotic bowel segments on serial radiographs that indicated fixed intestinal loops in addition to persistent metabolic acidosis, shock and persistent severe thrombocytopenia were recognized as the criteria for surgical intervention. During surgical intervention, macroscopic assessment of the abdomen showed various degrees of NEC signs, including necrotic changes, pneumatosis intestinalis, fragility, decreased tissue integrity, edema and discoloration. After surgical intervention, histopathological evaluation of the excised intestinal tissues was performed for the findings of NEC.

Patients diagnosed with true NEC were defined as the study group, and blood samples were taken from these patients and collected into serum test tubes (Minicollect® 1cc, Grenier Bio-one, Kremsmünster, Austria) for evaluation of SAA, C5a, CRP, and IL-6 serum levels at the time of diagnosis (1st day), the 3rd day, and the 7th day after the diagnosis. Infants at ≤32 weeks of gestation and ≤1,500 g of birth weight, and without signs of NEC were allocated to the control group, and their blood samples were collected for evaluating serum levels of SAA, C5a, CRP, and IL-6 at the 3rd day of life.

Serum CRP levels were measured with the nephelometric method (sensitive value = 0.8 mg/dl) (CRP kit, Roche, Germany) (IMMAGE device, the Beckman-Coulter, USA). IL-6 levels were measured with a solid phase enzyme labeled chemiluminescent immunometric assay (IL-6 kit, Siemens Healthcare Products Ltd, Hanbers, USA) (sensitivity value = 2 pg/ml) (Immulite 2000 device, USA), and values were recorded. Serum amyloid-A (In vitrogen, Hu SAA, immunoassay kit, Camarillo, CA, USA, Cat. No. KHA0012) (sensitive value = 4 ng/ml); and C5a levels (BD™ Human OptEIA C5a ELISA Kit II, San Jose, CA, USA, Cat. no. 557965) (sensitive value = 0.047 ng/ml) were measured with an ELISA method (ELISA washer-reader device, Biotech, USA), and values were recorded.

**Statistical analysis**

Statistical analysis was performed with the SPSS 15.0 program (Chicago, IL, USA). The normal distribution of variables was tested with the Shapiro-Wilk test. Descriptive statistics were given as mean and standard deviation (SD) or median and interquartile range (IQR); categorical variables were given as values and percentage. ANOVA with Bonferroni was used for intergroup analyses for parametric variables, however the Kruskal-Wallis test and the Mann-Whitney U test were used for intergroup comparisons of nonparametric variables. Bonferroni adjustment was used for different comparisons. Chi-square test was used to compare categorical variables for independent groups. To compare dependent groups, the Friedman test and the Wilcoxon test with Bonferroni adjustment were used. Correlation was performed by Spearman correlation analysis. Logistic regression analysis was used to predict perforation and mortality risk. Perforation occurrence and factors affecting the mortality of the infants were considered as the dependent factors, while SAA, C5a, CRP, IL-6 were recognized as the independent factors. Receiver-operating characteristic (ROC) analysis was performed for cut-off values of prediction of intestinal perforation and death. Values of $P < 0.05$ were considered significant.
Results

Comparison of the control and the NEC groups

During the study period, 22 of 187 patients (11.7%) were diagnosed with true NEC, and allocated to the study group. The selection of the control group was performed by a simple randomization method using a table of random numbers to select 23 patients as the control group among 187 patients without NEC. No significant differences were found between the study and the control groups in terms of gestational age, modes of delivery, gender, birth weight, APGAR scores, need for resuscitation, antenatal steroid treatment, maternal chorioamnionitis and mechanical ventilation therapy ($P > 0.05$) (Table 1). In the NEC group, the serum levels of SAA, C5a, CRP and IL-6 were found to be significantly higher than in the control group on the 1st, 3rd and 7th days of diagnosis ($P < 0.001$). C5a levels were found to be similar at the time of diagnosis, and on the 3rd and 7th days of the NEC episodes. However, CRP and IL-6 levels showed an increase on the 3rd and 7th days of NEC compared to the 1st day. In contrast, although SAA levels were significantly higher at the time of diagnosis, these levels were decreased on day 3 and day 7 (Table 2).

Evaluation of infants with stage II and III of NEC

Ten patients (45.5%) had stage II NEC and 12 patients (54.5%) had stage III NEC. There was no significant difference between NEC stages in terms of demographic variables ($P > 0.05$). There were no significant differences between infants with stage II and III NEC in terms of SAA, C5a, CRP and IL-6 serum levels at the time of diagnosis (1st day), the 3rd day, and the 7th days of diagnosis ($P > 0.005$) (Table 3). C5a levels were also similar between infants with stage II and III NEC on the 1st, 3rd and 7th days. Serum IL-6 levels increased from the 1st day to the 3rd and 7th days. CRP levels showed an increase from the 1st day to the 3rd day, then showed a decrease at the 7th day, in both stage II and stage III NEC patients. Although serum SAA levels were higher at the time of diagnosis, they decreased in both stage II and III NEC patients on day 3 and day 7.

Evaluation of infants with surgical intervention, and survivals

Ten patients (45.5%) with stage III NEC underwent surgical intervention due to intestinal perforation, and eight patients (36.4%) with stage III NEC died. There was no significant difference between the infants who survived and the ones who died in terms of demographic variables ($P > 0.05$). However, the birth weights of the infants who died [1,020 (260) g] were lower than those of the infants who survived [1,160 (385)] ($P = 0.025$). No statistically significant difference was found between patients with and without surgical intervention in terms of demographic variables ($P > 0.05$). However, the gestational age of infants who underwent surgical treatment (27.2 ± 1.6 versus 29.0 ± 2.1 weeks) was lower ($P = 0.02$), and these infants were found

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control ($n = 23$)</th>
<th>NEC ($n = 22$)</th>
<th>$P$</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Mean ± s.d.</td>
<td>Mean ± s.d.</td>
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<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
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<tr>
<td>Gender</td>
<td></td>
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<tr>
<td>Boy</td>
<td>11 (47.8%)</td>
<td>12 (52.2%)</td>
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<tr>
<td>Girl</td>
<td>12 (54.5%)</td>
<td>10 (45.5%)</td>
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<tr>
<td>Birth weight (g)</td>
<td>1,120 (350)</td>
<td>1,115 (342.5)</td>
<td>0.80</td>
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<tr>
<td>Gestational age (week)</td>
<td>28.5 ± 0.5</td>
<td>28.4 ± 0.4</td>
<td>0.53</td>
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<td>Modes of delivery</td>
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<td>NVD</td>
<td>8 (47.1%)</td>
<td>9 (52.9%)</td>
<td>0.76</td>
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<td>C/S</td>
<td>15 (53.6%)</td>
<td>13 (46.4%)</td>
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<td>APGAR 1. min.</td>
<td>5 (2)</td>
<td>4 (2)</td>
<td>0.39</td>
</tr>
<tr>
<td>APGAR 5. min.</td>
<td>7 (3)</td>
<td>6.5 (1)</td>
<td>0.39</td>
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<td>Mothers’ age (year)</td>
<td>29.2 ± 0.8</td>
<td>27.7 ± 1.4</td>
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<td>Resusitation</td>
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<td>Performed</td>
<td>14 (60.8%)</td>
<td>16 (72.7%)</td>
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<td>Antenatal steroid</td>
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<tr>
<td>Performed</td>
<td>17 (73.9%)</td>
<td>17 (77.3%)</td>
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<td>Maternal infection</td>
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<tr>
<td>PDA</td>
<td>5 (21.7%)</td>
<td>8 (36.3%)</td>
<td>0.34</td>
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<tr>
<td>RDS</td>
<td>15 (65.2%)</td>
<td>15 (68.2%)</td>
<td>1.0</td>
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<tr>
<td>ICH</td>
<td>17 (73.9%)</td>
<td>22 (100%)</td>
<td>0.003*</td>
</tr>
<tr>
<td>PDA and RDS</td>
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<td>5 (22.7%)</td>
<td>0.29</td>
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<td>4 (17.4%)</td>
<td>10 (45.5%)</td>
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<td>Mechanical ventilation therapy</td>
<td>7 (30.5%)</td>
<td>4 (18.2%)</td>
<td>0.27</td>
</tr>
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</table>

* Values of $P < 0.05$ were considered significant.
ICH, intracerebral hemorrhage; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus.
to have more need for resuscitation ($P = 0.046$) compared to the other infants. Also, the mortality rate of infants who underwent surgical intervention (6 of 10 patients) was significantly higher than that in infants without surgical intervention (1 of 12 patients) ($P = 0.007$).

C5a levels at the time of diagnosis ($P = 0.039$) and at the 3$^{rd}$ day of diagnosis ($P = 0.047$) were significantly higher in the patients who died than in those who survived. However, there were no significant differences related to the other parameters (CRP, IL-6 and SAA) on the other days (Table 4) between the patients who survived and the patients who died. There were no significant differences between patients who were treated surgically and those who were not treated surgically with respect to serum levels of SAA, C5a, CRP, or IL-6 at the 1$^{st}$ [SAA: 583.43 (488.48) vs. 531.39 (634.52); C5a: 7.86 (1.29) vs. 7.70 (0.55); CRP: 16.20 (25.73) vs. 27 (25.70); IL-6: 78.40 (554.10) vs. 56 (181.50)], the 3$^{rd}$ [SAA: 406.77 (482.56) vs. 452.45 (430.57); C5a: 7.69 (0.52) vs. 7.34 (0.91); CRP: 47 (57.20) vs. 34.80 (181.50); IL-6: 244 (627.45) vs. 129 (50.50)] and the 7$^{th}$ [SAA: 467.35 (412.06) vs. 389.72 (358.44); C5a: 7.84 (0.82) vs. 7.64 (0.44); CRP: 63.70 (49.15) vs. 45.90 (26.25); IL-6: 286 (394.50) vs. 234.50 (246)] days of diagnosis of NEC ($P > 0.005$).

Predicting requirement for surgery and mortality

Logistic regression analysis was performed for SAA, C5a, and CRP, and IL-6 levels for the prediction of perforation and requirement for surgery. The AUC for SAA (0.843) and C5a (0.833) were significantly higher than those of CRP (0.687) and IL-6 (0.657) at the time of diagnosis for predicting perforation in infants with NEC ($P = 0.02$). There were no significant differences in terms of AUC on day 3 [SAA: 0.750, C5a (0.802), CRP (0.824), IL-6 (0.847)] and day 7 [SAA (0.826), C5a (0.806), CRP (0.844), IL-6 (0.861)] among SAA, C5a, CRP and IL-6 for predicting perforation in these infants ($P > 0.05$) (Fig. 1). Similarly, the AUC for C5a (0.876) and SAA (0.820) were
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significantly higher than those of CRP (0.686) and IL-6 (0.709) at the time of diagnosis for predicting mortality in infants with NEC ($P = 0.02$); however, no significant difference was determined between the AUC for C5a and SAA ($P > 0.05$). There were no significant differences among the AUC values for these markers at both day 3 [C5a (0.855), SAA (0.793), CRP (0.773), and IL-6 (0.844)] and day 7 [C5a (0.827), SAA (0.863), CRP (0.795), and IL-6 (0.816)] for determining mortality risk in these infants ($P > 0.05$) (Fig. 2).
Discussion

To the best of our knowledge, this is the first study to evaluate the efficacy of C5a and compare it to that of CRP, IL-6 and SAA in the diagnosis and follow-up of NEC in premature infants. All four parameters were found to be higher at the time of the diagnosis. However, it was found that C5a levels did not change on the 3rd and 7th days of NEC and stayed higher in the follow-up of the disease. On the other hand, the levels of CRP and IL-6 continued to increase during the disease process, and SAA levels were shown to decrease on the 3rd and 7th days of diagnosis. No differences were found between stage II and III NEC in terms of these four parameters. These four parameters were also evaluated for predicting perforation and death in these patients, and C5a and SAA levels were found to be significantly higher than CRP and IL-6 levels at the time of diagnosis in the patients with NEC. Therefore, these parameters are suggested as useful markers for the prediction of patients requiring surgery. However, C5a and SAA were not superior to each other for predicting surgery requirement in terms of sensitivity, specificity and AUC. Although no significant differences were determined between C5a and SAA, C5a was found to be slightly superior to SAA for the prediction of death at the time of diagnosis. SAA levels on day 3 and 7 of NEC were found to be significantly higher in the patients who died, suggesting that patients with high SAA levels, not responding to treatment, might result in higher rates of mortality.

Hypoxic-ischaemic injury to the gastrointestinal (GI) tract is believed to be a major contributing and potentially inciting factor in the development of NEC (Lin et al. 2008; Thompson and Bizzarro 2008; Schnabl et al. 2008). Ischemia-reperfusion (I/R) injury models in animals indicated that complement activating products like C5a have a key role in intestinal damage (Fleming et al. 2003; Arumugam et al. 2006). In addition, animals subjected to intestinal I/R injury who were treated with complement inhibitor (C5a monoclonal antibody and C5a receptor inhibitors) showed attenuation of histological mucosal injury, reduction in vascular permeability, edema and hemorrhage in intestinal tissue, and increased survival rate (Arumugam et al. 2002, 2006; Fleming et al. 2003, 2008). These data suggest that C5a is an important factor for the mucosal damage in the pathogenesis of NEC. Another animal model of sepsis revealed that C5a receptors are increased in different tissues such as the liver, kidney and myocardium in response to high plasma levels of IL-6 (Riedemann et al. 2002). Animal models of sepsis in which IL-6 blockers were used for the treatment of animals, resulted in a reduction of C5a receptor expression and increased survival rate (Riedemann et al. 2002, 2003). Also, animals with sepsis that were treated with C5a receptor inhibitors showed a reduction in IL-6 serum levels (Riedemann et al. 2003). Interestingly, C5a was shown to be activated within the first hour of stimulation, and the IL-6 surge was observed six hours into the stimulation. Furthermore, it was reported that IL-6 production from mononuclear cells and polymorph nuclear leukocytes in the circulation was triggered by C5a (Riedemann et al. 2003). This data suggests that C5a increases in early phases of NEC and has a regulatory role for the production of IL-6. In correlation with this data, we showed that C5a can be applied as a useful marker in NEC as it showed an increase at the time of diagnosis.

SAA and CRP are rapid acting acute phase proteins produced by hepatocytes (Malle and De Beer 1996; Lannergard et al. 2008). IL-6 is an important cytokine trig-
growing production of these acute phase proteins from the liver (Jiang et al. 1995; Malle and De Beer 1996). SAA and CRP were also reported to increase by 1 to 1000 fold, triggered by IL-6 during the infection period and SAA levels were reported to be correlated with the amount of tissue destruction (Schultz and Arnold 1990; Jiang et al. 1995; Malle and De Beer 1996). Additionally, synthesis and release of CRP and SAA are genetically controlled and regulated by IL-1, TNF-α and IL-6 (Malle and De Beer 1996; Lannergård et al. 2005). Although some studies suggest that CRP and SAA increase in parallel to each other, SAA was shown to be a more sensitive inflammatory marker than CRP (Malle and De Beer 1996). Moreover, SAA was suggested to be a more useful and sensitive marker than CRP for diagnosis and follow-up of bacterial and viral infections due to its rapid and early surge form even minimal inflammatory stimulation (Riedemann et al. 2002). In a recent study, it was reported that SAA was a more accurate, safe and reliable marker for the diagnosis and follow-up of neonatal sepsis than CRP, and is especially useful at the onset of inflammation for rapid diagnosis of sepsis (Cetinkaya et al. 2009). As indicated in our study, SAA showed an early increase at the time of diagnosis, suggesting that SAA may be a more useful inflammatory marker than CRP for NEC.

In conclusion, this is the first study that has shown the efficacy of C5a in the diagnosis of NEC. This study also suggests that C5a may be useful for predicting perforation and death in premature infants with NEC. As far as we know, there is only one study that evaluated the levels of complement system components such as C3, C4, and C5 in preterm infants with NEC, and concluded that the pathogenesis of NEC may not involve primarily complement activation (Stevenson et al. 1980). However, the results of this study indicate that C5a might be considered as an important contributing inflammatory factor in NEC. This is due to the fact that it increases rapidly and significantly at the onset of inflammation, and triggers production of other inflammatory markers including IL-6, SAA and CRP. Our study also showed that all four parameters were significantly higher at the time of diagnosis of NEC. Additionally, SAA levels were significantly higher at the time of diagnosis and then decreased during follow-up. As a result, we suggest that C5a and SAA seem to be superior to CRP and IL-6 in both diagnosis and follow-up of NEC. Therefore, it may be reasonable to use these markers in combination with clinical and radiographical findings in NEC. Further studies with a greater number of premature infants are required to support the use of various markers in NEC.

Conflict of Interest

We have no conflict of interest.

References


