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Original Article

Can immature reticulocyte fraction be an inflammatory biomarker in late-preterm infants diagnosed with congenital pneumonia?

Ilkay Er[1], Ceren Cetin[2], Canan Baydemir[3]

[1]Department of Neonatology, Health Sciences University, Kocaeli Derince Training and Research Hospital, Kocaeli, Turkey
[2]Department of Pediatric Infection Disease, Health Sciences University, Kocaeli Derince Training and Research Hospital, Kocaeli, Turkey
[3]Department of Bioistatistics and Medical Informatics, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey

Abstract

Aim: Congenital pneumonia (CP) is serious respiratory infection of the neonates. Recently introduced hematological parameter, immature reticulocyte fraction (IRF), has been investigated to gather clinical information about the prognosis of anemia as well as to measure the level of inflammatory activity in adult patients. In this study of late-preterm infants diagnosed with CP, we compared IRF with common sepsis biomarkers and evaluated its role as inflammatory biomarker in neonates.

Material and Methods: Late-preterms were categorized based on infectious vs. non-infectious etiology of the respiratory distress. Blood samples were taken at 48-72 hours after birth. IRF was measured with Sysmex XN-3000. Biomarkers such as complete blood count parameters, C-reactive protein (CRP), and procalcitonin (PCT) were used for the comparison.

Results: Total of 25 late-preterms, 14 in CP-group and 11 in transient tachypnea of the newborn (TTN) group were included study. The groups were comparable in terms of gestational age, birth weight and cesarean section rate. The proportion of prolonged membrane rupture was significantly higher in CP-group. No significant differences were found between hemoglobin, hematocrit and reticulocyte in both groups (p>0.05). The value of IRF was higher in CP-group compared to in TTN-group, although it was not statistically significant (37.8±7.2% vs. 31.6±9.4%, respectively) (p=0.08). PCT was significantly higher in CP-group (p=0.017). No differences were found in other biomarkers between the groups (p>0.05).

Conclusion: Our results suggest that PCT can be diagnostic marker in CP, but further studies are needed to confirm role of IRF in neonatal inflammation.

Keywords: Biomarker; immature reticulocyte fraction; neonate

INTRODUCTION

Pneumonia represents a global child health problem associated with high morbidity and mortality, especially in neonatal period (1,2). Despite recent advances in management strategies, rapid and accurate diagnosis is major challenge. Subtle clinical features, especially in preterm infants, and paucity of specific and early objective diagnostic evaluations may contribute to a delay in initiation of treatment (3).

Depending on the time of manifestation of infection, early-onset pneumonia is defined for pneumonias in the first week of life. Congenital pneumonia (CP) is associated with prolonged premature rupture of membranes, chorioamnionitis and other perinatal maternal infections, and usually presents within first day of life. CP can be accepted as a subgroup of early-onset pneumonia (2,3). Pneumonia is common presentation of early-onset sepsis (EOS) of the neonates (4).

Common septic diagnostic parameters include laboratory tests, such as complete blood count (CBC) parameters, absolute neutrophil count (ANC), immature/total neutrophil (I/T) ratio, C-reactive protein (CRP), and procalcitonin (PCT), which are nonspecific and often elevated also due to non-infectious respiratory condition such as transient tachypnea of the newborn (TTN) and respiratory distress syndrome (RDS) (3-7). Conventional blood culture is the most helpful diagnostic test, whereas an etiologic agent may not be identified in many cases (3).

Advances in diagnostic hematological tests allow use of novel CBC parameters such as the immature reticulocyte fraction (IRF) for diagnostic purposes. This parameter not only shows the fraction of immature reticulated young cells

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Corresponding Author: Ilkay Er, Department of Neonatology, Health Sciences University, Kocaeli Derince Training and Research Hospital, Kocaeli, Turkey **E-mail:** ilkayer7778@yahoo.com

that have been recently released into blood circulation, but also the status of erythropoiesis in bone marrow (8,9). In a recent study, IRF has been investigated to gather clinical information about the prognosis of anemia as well as to measure the level of inflammatory activity (10). However, best of our knowledge, there is no study on IRF as an inflammatory biomarker in neonates.

Late-preterm infants represent a growing population among the overall population of preterm infants. They are often considered by clinicians as physiologically similar to full-term infants, whereas the risk of early sepsis and respiratory morbidity is higher in late-preterms (11,12).

In this study of late-preterm infants diagnosed with CP, we compared the new hematological parameter IRF with common sepsis biomarkers and evaluated the role of IRF as a new inflammatory biomarker in neonates.

MATERIAL and METHODS

Study design

This prospective study was conducted between February and August 2018 at neonatal intensive care unit (NICU), Kocaeli Derince Training and Research Hospital. The study protocol was approved by local ethics committee of Kocaeli University (KIA 2018/20). Late-preterms admitted to NICU were enrolled into the study after obtaining written informed consent from parents. Detailed information on prenatal history was recorded at admission.

Late-preterms were categorized based on respiratory distress either infectious (i.e. CP) or non-infectious (i.e. TTN), and the groups were referred as the CP-group and TTN-group, respectively. Infants with other respiratory disorders, especially those with RDS receiving surfactant, as well as those with congenital or chromosomal abnormalities, perinatal asphyxia, intracranial hemorrhage, disseminated intravascular coagulation, hemolysis and any other anemia etiology were excluded from the study.

In the study, late-preterm infants were defined as infants born from 34 through 366 weeks of gestation (11). Infants with the history of maternal infection were evaluated for the sepsis with following laboratory parameters; total leukocyte count (WBC)<5.000/mm^3, ANC<1.000/mm^3, I/T ratio≥0.2, platelet count <100.000/mm^3 or CRP>5 mg/L or PCT>0.5 ng/mL (6,7). Anemia was defined for a hemoglobin (HGB) or hematocrit (HCT) at or below the 2.5th percentile for gestational age (13).

Perinatal asphyxia was defined for the late-preterms according to the guidelines of the American Academy of Pediatrics and the American College of Obstetrics and Gynecology in the presence of an acute perinatal event with the following criteria: 1) Apgar score <5 in 5 minutes and 10 minutes; 2) fetal umbilical artery pH <7.0, or base deficit ≥12 mmol/L, or both; 3) neurological sequelae (e.g., seizures, coma, hypotonia); and/or 4) multiple organ involvement consistent with hypoxic-ischemic encephalopathy (14).

In the study, CP was defined as respiratory distress appearing within 72 hours of birth, with radiological features of pneumonia as reported by a radiologist (bilateral dense alveolar densities with air-bronchograms, patchy segmental densities, granular densities), with at least one risk factor for prenatal infection (maternal fever>38°C, suspected or confirmed chorioamnionitis, urinary tract infection, prolonged rupture of membranes>18 hours, spontaneous preterm labour) or elevated acute phase reactants or positive blood culture for a proven pathogen (2,3,15,16).

The diagnosis of TTN was made with following criteria: 1) respiratory rate higher than 60 per minute within six hours after delivery, grunting sounds with breathing, flaring of the nostrils, retractions; 2) tachypnea lasting for at least 12 hours; 3) chest x-ray indicating at least one of the following: increased aeration, flattening of the ribs, fluid accumulation in the interlobar fissures and costophrenic angle, vascular congestion and depression of the diaphragmatic domes or increased anteroposterior diameter or both; 4) exclusion of either known respiratory (RDS, pneumonia, meconium aspiration, congenital heart diseases) or non-respiratory disorders (neurologic, metabolic, hematologic causes) likely to cause tachypnea (17).

Blood samples

Blood samples were collected at 48-72 hours after birth simultaneously in ethylenediaminetetraacetic acid (EDTA) tubes (for CBC and IRF) as well as in plain tubes (for CRP and PCT). Peripheral smears were prepared for determining I/T ratio, and blood cultures were obtained in Bactec culture bottles before administration of antibiotics.

The routine hematological investigations performed in EDTA tubes on a multi-channel automated cell counter included CBC indices such as total leukocyte count (WBC), red blood cell count, HGB, HTC, ANC and platelet count. And normal reference ranges for CRP and PCT derived using specific kits were 0-6 mg/L and 0-0.05 ng/mL as per our laboratory, respectively.

Measurement of IRF

The collected 2-3 ml blood samples in EDTA tubes for IRF were kept at room temperature for 4-6 hours before being sent to Hematology Laboratory for analyses. They were identified by flow cytometer techniques using a nucleic acid specific fluorescent dye in reticulocyte channel of automated hematology analyzers (Sysmex XN-3000; Sysmex Corporation, Japan). This dye penetrates through the cell membrane and stains the RNA in immature reticulocytes. This automated system provides a graphical image of different populations based on size and quantity of RNA and establishes the reticulocyte fractions. IRF consists of combined fraction of medium and high fluorescence reticulocyte (8,18).

Statistical analysis

IBM SPSS 20.0 (IBM Corp., Armonk, NY, USA) statistical programs were used to analyze data. Normality was tested using Kolmogorov-Smirnov test. Variables were expressed as mean±standard deviation (SD), median (25th-75th percentiles), and counts (percentages). Comparisons

of variables between the groups were done using the Student's t, Mann-Whitney U, Monte Carlo and Fisher's Exact Chi-Square test. For the comparison between the time-points, paired samples t test were used. A p value <0.05 was considered statistically significant. It was accepted as "α = 0.05, 1-β (Power) = 0.70 and effect size = 0.9" for the power analysis of study, and the minimum number of patients were determined as totally 26 patients, 13 in each group.

RESULTS

A total of 25 late-preterm infants (13 female and 12 male), were included in this study, with a median gestational age and mean birth weight of 35.4 (range, 34.1-37) weeks and 2570±586.3 g, respectively.

While the rate of prolonged rupture of membrane was significantly higher in CP-group (p=0.001), two groups had similar rate of maternal urinary tract infection and use of antenatal prophylactic cefazolin (p>0.05).

Two groups were initiated empirical antibiotic treatment (ampicillin and gentamicin) at the admission. The median duration of antibiotic use and hospitalization were longer in CP-group (p=0.042 and p=0.044, respectively). No growth in first blood cultures were detected in both groups. The characteristics of the late-preterm groups were represented in Table 1.

While the mean HGB, HTC and reticulocyte were 18.48±2.01 g/dl, 53.8±2 % and 3.2±1 %, respectively, in CP-group, these values were 18.29±2.53 g/dl, 53.3±3 %, and 3.1 ±1 %, respectively, in TTN-group.

Table 1. The characteristics of late-preterm groups, mean±SD/median (25^{th}-75^{th})/n(%)

	TTN group (n:11)	CP group (n:14)	p
Gestational weeks, median (25^{th}-75^{th})	**36** (35.1-37)	**35.3** (34.1-36.2)	0.189[a]
Birth weight (g), mean± SD	**2626**±426.5	**2526**±700.2	0.141[b]
Caesarean section, n(%)	**12** (75)	**11** (78.6)	0.435[c]
Maternal urinary tract infection, n(%)	**12** (75)	**11** (78.6)	0.921[c]
Prolonged rupture of membranes, n(%)	**2** (12.5)	**14** (100)	**0.001**[c]
Antenatal prophylactic cefazolin, n(%)	**15** (93.8)	**13** (92.9)	0.852[c]
Antibiotic usage period (days), median(25^{th}-75^{th})	**3** (2-4)	**7** (5-10)	**0.042**[a]
Hospitalization period (days), median(25^{th}-75^{th})	**6** (5-7)	**11** (8-15)	**0.044**[a]

TTN: Transient Tachypnea Of The Newborn, CP: Congenital Pneumonia
[a]: Mann Whitney U test; [b]: Student's t test; [c]: Chi Square test

Table 2. The results of common biomarkers, blood count parameters and IRFs in TTN group and CP group, mean±SD/median(25^{th}-75^{th})

	TTN group (n:11)	CP group (n:14)	p[a,b]
WBC (/mm^3)	17.542±6.797	13.700±4.193	0.144[b]
HBG (g/dl)	18.29±2.53	18.48±2.01	0.806
HTC (%)	53.3±3	53.8±2	0.750[b]
Platelet (/mm^3)	280.000 (227.000-310.000)	279.000 (229.000.299 500)	0.835[a]
ANC	7.755±2.441	6.552±2.434	0.314[b]
I/T ratio	0.07±0.04	0.09±0.04	0.190[b]
CRP (mg/L)	0.1 (0.1-4.5)	1.1 (0.1-6.5)	0.245[a]
PCT (ng/mL)	0.71 (0.27-3.06)	9.11 (0.65-17.52)	**0.017**[a]
Reticulosit (%)	3.1±1	3.2±1	0.740[b]
IRF (%)	31.6±9.4	37.8±7.2	0.080[b]

TTN: Transient Tachypnea Of The Newborn, CP: Congenital Pneumonia, WBC:White Blood Cell, HBG: Hemoglobin, HTC: Hematocrit , ANC: Absolute Neutrophil Count, I/T: Immature/Total Neutrophil Ratio, CRP: C-Reactive Protein, PCT: Procalcitonin, IRF: Immature Reticulocyte Fraction
[a]: Mann Whitney U test, [b]: Student's t test

No significant differences were found between the mean HGB, HTC and reticulocyte for late-preterm infants in both groups (p>0.05).

The value of IRF was higher in CP-group compared to in TTN-group, although it was not statistically significant (37.8±7.2 % vs. 31.6±9.4 %, respectively) (p=0.08).

In CP-group, PCT was found significantly higher than in TTN-group (p=0.017). No differences were found in other biomarkers between the groups (p>0.05).

Table 2 shows the results for biomarkers and IRFs in the groups.

DISCUSSION

In our study, we observed significant higher PCT value as well as higher IRF value in late-preterm infants diagnosed with CP compared to in those diagnosed with TTN. However, the IRF was not revealed statistically significant.

Pneumonia is a serious respiratory infection of the neonates. Timely diagnosis and rapid treatment of CP is very crucial with the possibility of life-threatening complications. Maternal Group B Streptococcal colonization and chorioamnionitis are important risk factors. Pneumonia results from aspiration of infected amniotic fluid during intrauterine period or during the birth process causes. Onset may be within hours of birth and part of an early sepsis that confined to the lungs. Signs may be limited to respiratory distress or progress to persistent pulmonary hypertension, septic shock and death. Diagnosis is by clinical and laboratory evaluation for the sepsis. However, clinical signs are often nonspecific in neonates and mimicking a range of non-infectious respiratory diseases. Chest X-ray is should be performed for any infants with respiratory distress. The most common abnormality identified for pneumonia is dense bilateral alveolar infiltrates with air-bronchograms. Treatment is initial broad-spectrum antibiotics changed to organism-specific drugs as soon as possible (2-5).

Late-preterm infants represent a growing population among the overall population of preterms and are currently estimated to account for more than 70% of all preterm births (11). In contrast with term neonates, postpartum hypoactivity, poor feeding with or without respiratory distress can be easily confused with sepsis in these infants. Therefore, antibiotic therapy is frequently initiated for presumed infections, on the basis of the concerns regarding potentially serious adverse outcomes of the sepsis. However, distinction between clinical signs of sepsis and prematurity is generally far from being clear-cut (11,12,19). In multicenter study, Wolkewiez et al. found that 69.0% of late-preterms admitted to NICU were evaluated for sepsis during the first 3 days of life, and the cumulative incidence of culture-proven EOS was 4.42 episodes per 1000 admissions. In that study, infants born to mothers who received antenatal antibiotics were less likely to have positive blood cultures (19).

The conventional bacteriological culture is widely used and the most helpful diagnostic test for the infections in neonates. However, it should be noted that number of factors may interfere with ability to cultivate a likely pathogen from suspected sites, such as pretreatment with antibiotics, which limit the in-vitro but not in-vivo growth (3). In present study, blood cultures were sterile, most likely due to antenatal used cefazolin. Therefore, CP diagnosis of our participants was mainly based on maternal history, clinical and laboratory findings.

Complete blood count indices such as WBC, ANC, I/T ratio, and platelet are widely used for diagnosis of neonatal sepsis. Studies reported that WBC <5000/mm^3, ANC <1000/mm^3 or <5000/mm^3, I/T ratio ≥0.2 and platelet <50.000/mm^3 were associated with culture-proven sepsis (6,20). However, none of them have been found sensitive enough to reliably predict sepsis in newborns due to the possibility of being within normal range in early stage of infection (3,21). In our study, those mentioned parameters were within in normal range and there were no significant differences between the groups in terms of them.

Since they have been limited diagnostic values, the use of conventional sepsis biomarkers to support the diagnosis of suspected infection, including pneumonia, remains controversial (3). The sensitivity of CRP is high, but the specificity is relatively low in early stage of infection. It was reported that the combination of CRP and positive PCT may increase the diagnostic accuracy from 39% to 92% for bacterial infections and serial PCT testing may also reduce duration of antibiotic therapy (22,23).

PCT is acute phase reactant produced by monocytes and hepatocytes independent of circulating calcitonin levels. It concentrations increase within 2-4 hours after exposure to bacterial pathogen, peak at 6-8 hours during acute stage and remain elevated at least for 24 hours (7, 24). In a systematic review, the reported sensitivity and specificity of PCT in detection of neonatal sepsis were 72%-79% and 72%-90%, respectively (25). Lencot et al. used the cord blood PCT levels to distinguish infected infants from healthy ones with a cut-off value of 0.6 ng/ml. Infants whose cord blood PCT levels higher than 0.6 ng/ml were underwent diagnostic tests for evaluating early onset neonatal infection. As result of the study, they reported that their algorithm significantly reduced blood samples and antibiotic treatment (26). The usefulness of PCT in identifying bacteremia among neonates with pneumonia was also reported (27,28). Bozkaya et al. indicated the PCT as a good marker in CP with its high specificity and a high negative predictive value (88.8% and 90%, respectively) (28). In our study, supporting the previous study, PCT was found significantly higher in late-preterm infants diagnosed with CP compared to in those diagnosed with TTN.

The IRF is a new hematological parameter representing the ratio of immature form of red blood cell that have been recently released into blood circulation with high-RNA-content detected using automated hematological analyzers. It provides more precise evaluation of red cell

production, allowing near time estimation of erythropoiesis (8,9,18). Similarly, immature platelet fraction (IPF) is an immature stage of thrombocytopoiesis. Recently, Enz Hubert et al. have investigated whether IRF and IPF can also provide clinical information regarding the inflammatory activity and prognosis of sepsis. They evaluated adult patients, 12 with severe sepsis, 11 with non-complicated sepsis (sum of septic patient = 23), 14 with sterile inflammation (SIRS), and 173 healthy individuals. Despite similar platelet and absolute reticulocyte counts, a significant increase in both IRF and IPF were observed in 23 septic patients compared to healthy individuals (IRF in septic patient; 10-20% vs IRF in healthy individuals; <10%, p<0.0001) (10). In our study, we found also a higher IRF value in infectious CP-group vs in non-infectious TTN-group, but it was not statistically significant (37.8±7.2% vs 31.6±9.4%, respectively, p=0.08).

Besides from a well-known negative impact of acute inflammation on the erythropoiesis, it has been suggested that there is a positive relationship between sepsis and erythropoiesis: While steady-state erythropoiesis is seen in the bone marrow, stress erythropoiesis occurs in the spleen and liver. Hypoxia and acute anemia cause stress erythropoiesis to restore a sufficient number of red blood cells (29,30). Bennett et al. showed that inflammation mediated by Toll-like receptors induced stress erythropoiesis in mice by stimulating erythrocyte phagocytosis by splenic macrophages (31).

In above mentioned study of Enz Hubert, IRF was not found significantly higher in patients with severe sepsis compared to non-complicated sepsis patient at the time of administration (20.6±15.4% vs 12.6±6.0%, p>0.05, respectively), while the value of severe septic patients' IPF was found higher in those comparison. On the other hand, IPF was not able to discriminate patient with sepsis from patients with SIRS, they observed a lower trend of IRF values in patients with SIRS compared to septic patients (p=0.052). Since their subgroup of SIRS patient consisted of mostly individuals with post-operative significant bleeding, they explained this result with a well-known first negative impact of acute inflammation on the erythropoiesis (10,29).

The IRF is helpful in management of adults and children with a variety of hematological disorders. However, the neonatal reference intervals of IRF are limited and they have been published recently. In Christensen's study, mostly consisted of infants born at 39-40 weeks, the IRF value at birth was found 36.2±5.6% (32). Schiza et al. evaluated 181 infants of 32 to 36 weeks' gestation, at 2 and 6 weeks and at 3, 6, 9, and 12 months for IRF values, and the IRF at 2 weeks was reported 29±9% in those born at 34 to 36 weeks' gestation (33). We observed that our results were comparable only with Christensen's study, and compared to this study, our IRF values were relatively higher in CP-group and lower in the TTN-group. IRF variations observed in studies have been found to be related to method and population differences (34). In both previous studies, it was mentioned that the IRF values decreasing by increased days or gestational age (32,33). In 2002, Redzko et al. also reported that the cord blood reticulocyte fractions were different in normal pregnancy compared to pregnancy complicated by chronic intrauterine hypoxia (35).

To the best of our knowledge, this is the first study to investigate whether IRF has a diagnostic value in neonatal inflammation. However, the present study has certain limitations. Due to ethical concerns, no blood samples from healthy neonates could be obtained for comparisons. In addition, the sample size was small and the blood cultures of participants were no growth. Therefore, the evaluation for IRF's role as an inflammatory biomarker was based on the infectious or non-infectious etiology of the respiratory distress in late-preterm infants.

CONCLUSION

In conclusion, we suggest that PCT can be diagnostic marker in CP, but further studies with larger sample size are needed to confirm role of IRF in neonatal inflammation or sepsis.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: The study protocol was approved by local ethics committee of Kocaeli University (KIA 2018/20).

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