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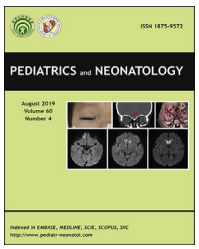

Original Article

Early diagnosis of necrotizing enterocolitis by plasma RELMβ and thrombocytopenia in preterm infants: A pilot study

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Key Words
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Background: As the inflammatory regulators, Resistin-like molecule β (RELMβ) and Resistin might be potential biomarkers of necrotizing enterocolitis (NEC), while thrombocytopenia is often related to the severity of NEC, clinical observation suggests that thrombocytopenia might be an early biomarker of NEC. The aim of this study was to evaluate whether RELMβ, Resistin and thrombocytopenia could be biomarkers for early diagnosis of NEC in preterm infants.

Methods: From January 2016 to March 2018, twenty-nine NEC preterm infants who were diagnosed with NEC (Bell's stage ≥II) by two independent neonatologists and twenty-nine non NEC preterm infants at neonatal intensive care unit in our hospital were enrolled in this case-control study. Preterm infants with a history of serious infections (sepsis, pneumonia), asphyxia, and congenital malformations were excluded from the study. The plasma RELMβ and Resistin were evaluated by enzyme linked immunosorbent assay (ELISA) and serum platelet levels were measured directly by ordinary light microscope at the diagnosis of NEC (Bell's stage ≥II).

Results: Plasma RELMβ levels in NEC group were significantly higher than control group ($P < 0.05$). The optimal cut-off value of RELMβ determined by receiver operating characteristic curve (ROC) was 378.3 ng/L. The overall estimates for sensitivity and specificity of high RELMβ concentrations in the detection of neonatal NEC were 71.4% and 91.7%, respectively. No

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significant difference was found in plasma Resistin levels between two groups ($P > 0.05$). If platelet level was less than 157×10^9/L, the sensitivity and specificity were 69.34% and 82.87%, respectively. Interestingly, the combination of RELMβ and thrombocytopenia increased sensitivity and specificity to 82.89% and 93.21%, respectively.
Conclusion: The combination of RELMβ and thrombocytopenia was a reliable biomarker for the early diagnosis of NEC in this study with 82.89% sensitivity and 93.21% specificity, respectively.

1. Introduction

Necrotizing enterocolitis (NEC) is the most common severe gastrointestinal emergency that affects newborns. The classic form of NEC occurs most commonly in preterm infants less than 34 weeks gestation, usually later than the first week after birth, and often occurs after the gastrointestinal (GI) tract becomes colonized with microbes.[1–4]

NEC often deteriorates within hours; thus early diagnosis is critically important in management of NEC and may have impact on outcome. However, early diagnosis of NEC is still difficult due to the lack of a fast and reliable biomarker or specific radiologic features. Several biomarkers, such as the intestinal fatty acid-binding protein (I-FABP), serum amyloid A (SAA), fecal calprotectin and various interleukins, proved to be helpful in the diagnosis of an ongoing NEC, since the levels of these biomarkers increase with the degree severity of disease.[5–7] However, since considering that each marker begins to elevate significantly and the time to normalization differs for each marker,[8,9] they are not useful in daily practice.

Resistin is a peptide hormone, belonging to the Resistin-like molecule family, which is a secreted protein rich in cysteine and encoded by *Resistin* (*RETN*) gene. It is also called adipose tissue-specific secretory factor and is found in inflammatory Zone 3 (FIZZ3).[10] In recent years, it has been reported that Resistin is a cytokine that promotes inflammatory response and its expression is significantly increased in sepsis and septic shock.[10,11] Additionally, increased circulating levels of Resistin are observed in inflammatory bowel disease.[12] Resistin-like molecule β (RELMβ)[13] also known as intestinal-specific resistance molecule, which maintains the functional barrier protein of gastrointestinal tract, was first found in mouse colon epithelial cells and it plays an important role in the local immunity of intestinal mucosa. It can protect the integrity of mucosal barrier by maintaining the function of intestinal epithelial cell barrier. Several studies disclosed RELMβ and Resistin to be inflammatory regulators; since NEC is an intestinal inflammatory disorder, we thus hypothesized that RELMβ and Resistin might be a potential biomarker in the early diagnosis of NEC. On the other hand, several studies reported that thrombocytopenia may be related to the severity of NEC,[14–16] and clinical observation disclosed that thrombocytopenia might be an early biomarker for early diagnosis of NEC.

According to the evidence above, this study aimed to evaluate whether RELMβ, Resistin and platelet could be potential biomarkers for the early diagnosis of NEC in preterm infants through a matched case-controlled observation study and the study aimed to define the sensitivity and specificity of these biomarkers in the early diagnosis of NEC.

2. Methods

2.1. Participants

This 1:1 matched case-control study was conducted in the neonatal intensive care unit (NICU) of Shenzhen Bao'an District Maternal and Child Health Care Hospital in China, which is a high-risk perinatal center with 1000 preterm infants per year. Eligible matched controls were identified by matching 1-to-1 to each NEC infant as age (±3 days), gestational age at birth (±1 week) and birth weight (±150 g).

From January 2016 to March 2018, twenty-nine NEC preterm infants who were diagnosed with NEC (Bell's stage ≥II) by two independent neonatologists and twenty-nine non-NEC preterm infants at the neonatal intensive care unit of our hospital were enrolled in this case-control study. Preterm infants with a history of serious infections (neonatal sepsis, pneumonia, etc.) and moderate-to-severe asphyxia, as well as those with congenital malformations in organs, including the brain, heart, gastrointestinal tract, kidney and respiratory tract, were excluded from this study.

Clinical chorioamnionitis was defined as fever ≥39.0 °C [102.2 °F] or 38.0 °C [100.4 °F] to 38.9 °C [102.02 °F] on two occasions 30 min apart, without another clear source PLUS one or more of the following: baseline fetal heart rate >160 beats/min for ≥10 min, excluding accelerations, decelerations, and periods of marked variability; maternal white cell count >15,000/mm^3 in the absence of corticosteroids and ideally showing a left shift (bandemia) and purulent-appearing fluid coming from the cervical os visualized by speculum examination.[17]

Moderate or severe asphyxia was defined as a pH of ≤7.0 or a base deficit of ≥16 mmol/L in a sample of umbilical cord blood or any blood obtained within the first hour after birth as well as one of the following: a 10-min Apgar score of <5 and ongoing resuscitation (e.g., assisted ventilation, chest compressions, or cardiac medications) initiated at birth and continued for at least 10 min.[18]

Antenatal steroids use was defined as mothers who received at least one complete course of antenatal

steroids. Breast feeding meant the preterm infants who were fed by maternal breast milk or donated breast milk. Ventilator use was defined as by the preterm infants who required mechanical ventilation or had a history of mechanical ventilation before the blood sample was drawn.

The protocol was approved by ethics committee of Bao'an district of Shenzhen (number: LLSC2015-12-31). Informed consent forms were given to the parents of all participants when there was sign of stage I NEC, on which was written stated that 2 ml of blood would be taken for research purposes.

2.2. Procedures

Two-ml venous blood samples were drawn from preterm infants within 6 h of the diagnosis of suspicious NEC (stage I) and then put into Ethylene Diamine Tetraacetic Acid (EDTA)

Table 1 Demographic and clinical characteristic of enrolled preterm infants ($\bar{x} \pm s$).

	Preterm infants with NEC (n = 29)	Preterm infants (n = 29)	*P* Value
Male, n (%)	17 (58.6)	18 (62.1)	0.788
GA (wk)*	33.5 ± 3.1	33.7 ± 3.2	0.397
BW (g)*	1758 ± 527.9	1764.7 ± 538.5	0.681
Age (d)	12.7 ± 5.3	13.2 ± 6.2	0.587
Apgar score (1min)	9.2 ± 0.4	9.1 ± 0.5	0.914
Apgar score (5min)	9.5 ± 0.7	9.4 ± 0.7	0.927
Chorioamnionitis, n (%)	6 (20.7)	7 (24.1)	0.753
Mild asphyxia, n (%)	5 (17.2)	5 (17.2)	1.000
Hypertension of pregnancy, n (%)	6 (20.7)	8 (27.6)	0.539
Antenatal steroids, n (%)	17 (58.6)	15 (51.7)	0.597
Breast feeding, n (%)*	29 (100)	29 (100)	1.000
Ventilator, n (%)	5 (17.2)	6 (20.7)	0.738
Surfactant, n (%)	7 (24.1)	8 (27.6)	0.764

*GA, gestational age. BW, birth weight. Breast feeding, breast milk, mother's own breast milk or donated breast milk.

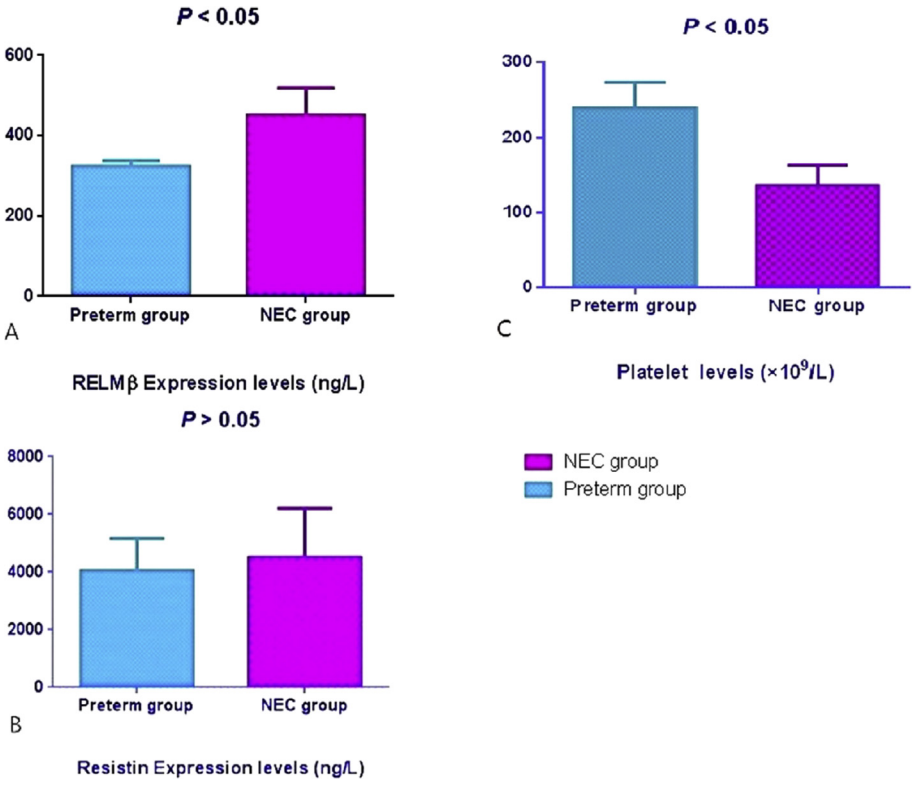

Figure 1 Serum platelet levels, plasma Resistin and RELMβ in the NEC and Control groups. A. Plasma RELMβ concentrations were significantly ($P < 0.05$) elevated in the NEC group (n = 29) compared with those in the Control group (n = 29). B. No significant difference ($P > 0.05$) was found in plasma Resistin concentrations between NEC group and Control group. C. Serum platelet counts were significantly ($P < 0.05$) decreased in the NEC group compared with those in the Control group.

anticoagulant tube. Samples were centrifuged for 5 min at 1500 r.min^{-1}. The plasma was frozen and stored at −7 °C until testing. No drugs were used before drawing blood samples.

After specimen collection was completed, we allowed the specimen to thaw naturally and reach room temperature. Plasma RELMβ and Resistin levels were measured by enzyme linked immunosorbent assay (ELISA). Serum platelet levels was measured directly by ordinary light microscope. Thrombocytopenia was defined as platelet count of less than 150 × 10^9/L.[13] The experimental protocol was carried out in strict accordance with the manufacturer's instructions.

2.3. Statistical methods

SPSS 18 statistical software was used to analyze data; data were described with mean ± standard deviation. We first tested the homogeneity of variance among the groups. If the variance was homogeneitic then we did the variance analysis; otherwise we did the non-homogeneity of variance analysis. The serum platelet levels, plasma levels of RELMβ and Resistin between both groups were compared by t test. ROC curve was used to determine the optimal cut-off value, sensitivity, specificity, positive predictive value, negative predictive value and the Youden index.

3. Results

3.1. General information

A total of 71 premature infants were diagnosed as stage I NEC, 29/40 premature infants developed into NEC (stage II), 29 non-NEC preterm infants were enrolled as control, and 13 premature infants could not be matched during the study period. There are no significant differences in age, sex, gestational age and birth weight between groups. Demographic and clinical data for the enrolled infants are summarized in Table 1.

3.2. The comparison of platelet levels, plasma levels of RELMβ and resistin expression in two groups

The plasma RELMβ levels in NEC patients were 450 ± 166.9 ng/L, (see Fig. 1) which were clearly higher than the non-NEC group (323 ± 83.9 ng/L), and the difference was statistically significant ($P < 0.05$). The serum platelet levels (129.9 ± 25.4 × 10^9/L) in NEC patients were significantly decreased compared to the non-NEC group (249.7 ± 39.5 × 10^9/L) ($P < 0.05$). No significant difference was found in Resistin levels in NEC patients compared with the non-NEC group (4522 ± 907 vs. 4048 ± 679 ng/L, $P > 0.05$).

3.3. The evaluation of RELMβ and platelet levels on the diagnosis of NEC

The optimal cut-off value of RELMβ determined by receiver operating characteristic (ROC) curve was 378.3 ng/L with sensitivity of 71.4% and specificity of 91.7% (Fig. 2). The positive predictive value, negative predictive value and the Youden index were 89.59%, 76.23%, and 0.631, respectively. The optimal cut-off value of platelet count was 157 × 10^9/L with 69.34% sensitivity and 82.87% specificity. The comparison of RELMβ and platelet levels on sensitivity, specificity, positive predictive value, negative predictive value and the Youden index are listed in Table 2.

4. Discussion

This matched case-control study showed that the sensitivity and specificity of high RELMβ concentrations in the detection of neonatal NEC were 71.4% and 91.7% respectively, with an area under the curve (AUC) of only 0.739. The combination of RELMβ and thrombocytopenia resulted a sensitivity of 82.9% and specificity of 93.2%, respectively. To the best of our knowledge, this was the first study that evaluated the combination of RELMβ and platelet levels as the potential biomarkers in the early diagnosis of NEC in preterm infants.

RELMβ is a cysteine-rich secreted protein that is highly similar to the crystalline structure of Resistin, another member of the family, mainly secreted by intestinal goblet cells.[19] McVay et al.[20] found that the enteritis induced by sodium glucomannan sulfate (DSS) was significantly reduced when compared with wild-type rats after the knock-down of *RELMβ* gene. In recent years, Chellappa et al.[21] also published a similar report. Further analysis was showed the

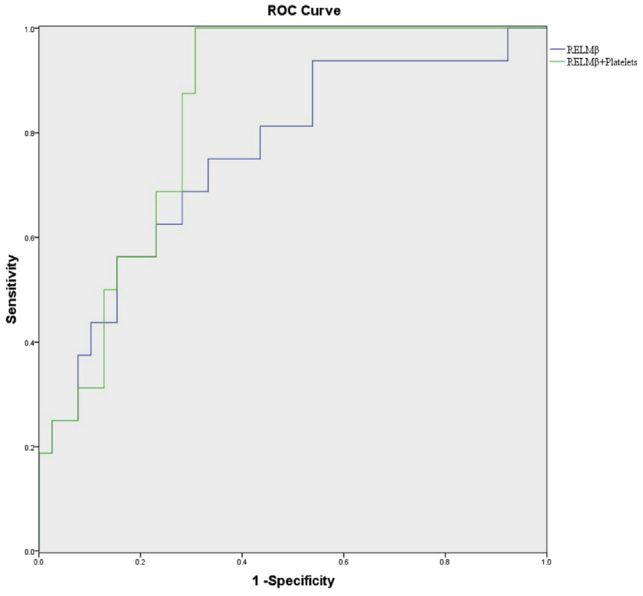

Figure 2 ROC curve for plasma RELMβ and combination RELMβ and Platelets in the NEC group. The blue line represents the ROC curve for RELMβ in the NEC group, the AUC was 0.739 ($P < 0.05$) and the cut-off level was 378.3 ng/L (sensitivity, 71.4% and specificity, 91.7%) in the NEC group. The green line represents the ROC curve for combination RELMβ and Platelets. The AUC was 0.841 ($P < 0.05$), the sensitivity and specificity increased to 82.89% and 93.21% respectively in the NEC group. ROC, receiver operating characteristic; RELMβ, resistin-like molecule β; NEC, necrotizing enterocolitis; AUC, area under the ROC.

Table 2 Comparison the parameters of each index.

Index	The optimal cut-off	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Youden index
RELMβ	378.3 (ng/L)	71.40	91.70	89.59	76.23	0.631
platelet	157 ($\times 10^9$/L)	69.34	82.87	77.62	70.98	0.522
RELMβ + platelet[a]	378.3 (ng/L)/157 ($\times 10^9$/L)	82.89	93.21	94.74	80.23	0.761

[a] Combination of 2 markers.

following: firstly, vitro and vivo experiments confirmed that RELMβ can directly stimulate macrophages to produce pro-inflammatory factors, such as tumor necrosis factor-α (TNF-α) and interleukin-15 (IL-15).[21] RELMβ-deficient macrophages significantly alleviated the activation of proinflammatory mediators in vitro, whereas rRELMβ-stimulated macrophages accelerated the production of proinflammatory cytokines.[22] Second, intraperitoneal injection of recombinant RELMβ molecules can cause the aggregation of neutrophils.[21] Finally, RELMβ is a bactericidal protein that promotes spatial segregation of the microbiota and the colonic epithelium.[23] Therefore, RELMβ plays an important role in anti-inflammatory regulation in the local immunity and infection of the intestinal tract. So far, however, no literature has reported the changes and clinical significance of RELMβ in neonatal NEC. Multiple experimental studies have found that infection can stimulate the secretion of Goblet specific factor RELMβ by intestinal epithelial cells. Bhinder et al. established the wild type rat model of enteritis by oral salmonella typhoid of rats and detected the levels of RELMβ hydrate mRNA in the cecum tissue after 1 day of infection. It was found that the levels of RELMβ protein were significantly higher than before infection.[24] In vitro, lipopolysaccharide (LPS) could induce the increase of RELM β mRNA expression in LS174T cells similar to Goblet cells.[25] It is suggested that RELMβ can be a good marker for early diagnosis of NEC.

The expression levels of RELMβ and Resistin in peripheral blood of NEC preterm infants were detected by ELISA, and the results showed the expression levels of RELMβ were significantly increased, while the expression levels of Resistin were increased, but the difference was not statistically significant. We speculate that this discrepancy arose due to the following mechanism: firstly, NEC is a gastrointestinal disease in neonates, which is associated with intestinal immunity, inflammatory response, etc, while RELMβ is an important intestinal immune and inflammatory factor. Secondly, several studies found that the enteritis induced by DSS was significantly reduced in RELMβ knockout rats.[20,21] Third, Resistin is a cytokine that promotes inflammatory response whose expression is significantly increased in sepsis and septic shock[11] but not in NEC; none of our NEC preterm infants developed sepsis, which is why Resistin was not expressed significantly in NEC preterm infants compared to the control group.

Our study did have some limitations. First of all, the small sample size enrolled from a single institution without reproduction was the limitation of this pilot study. Further study with more cases from different NICU's is needed to prove our findings. Also, the time of NEC onset was difficult to determine because it depended on the experience of caregivers. Therefore, the timing of blood sample drawn in each case cannot accurately reflect the time window of NEC onset. Despite this, we tried to get blood samples immediately when suspicious NEC occurred.

To sum up, the combination of newly molecular RELMβ with the very common biomarker of thrombocytopenia seemed to be the reliable biomarker for early diagnosis of neonatal NEC, and it might help in daily practice at NICU.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pedneo.2019.01.006.