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ORGINAL ARTICLE

CD64 Expression on Polymorph Nuclear Cells as a Sensitive and Specific Diagnostic Biomarker for Neonate Pneumonia

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SUMMARY

Background: **Neonatal pneumonia (NP) is one of the major causes of neonatal death. Current NP diagnosis depends on a detailed history, physical examination, and radiographic and laboratory findings. There is no specific biomarker or diagnostic indicator of NP.**
Methods: **In this study, we tried to find a reliable biomarker for quick NP diagnosis by collecting peripheral blood from neonates with NP and transient tachypnea of the newborn (TTN), and subsequently tested the expression of CD64 on white blood cells using flow cytometry. The cellularity of each blood cell population was also quantified. Furthermore, procalcitonin (PCT) and C-reactive protein (CRP) levels were evaluated in the blood sera.**
Results: **We found that NP patients had moderately increased polymorphonuclear cells (PMNs), as well as elevated PCT and CRP levels in the blood sera. Importantly, the expression of CD64 on PMNs was profoundly increased in NP patients but not TTN patients. The receiver operating characteristic (ROC) curve of PMN CD64 index suggests that PMN CD64 index is sensitive and specific for NP diagnosis.**
Conclusions: **Our study reveals that PMN CD64 could be a fast and reliable biomarker for NP diagnosis.**
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KEY WORDS

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INTRODUCTION

Neonatal pneumonia (NP) is one of the major causes of neonatal death, with the morbidity rate of 3.5 - 25% due to consequent septicemia and respiratory distress syndrome [1]. The risk factors of NP include maternal group B streptococcus carrier, chorioamnionitis, maternal fever, PROM, prematurity, perinatal depression [2]. The signs of pneumonia are non-specific in neonates, and quick diagnosis and therapies are often impossible so the mortality is high. Currently, there is no specific biomarker or diagnostic indicator of NP, and NP diagnosis depends on a detailed history, physical examination, and radiographic and laboratory findings. If a significant humoral or cellular change could be identified

in the peripheral blood of neonates suffering NP, it would help establish a quick method for NP screening. Transient tachypnea of the newborn (TTN) is another cause of respiratory distress in neonates and is triggered by insufficient lung fluid removal. A small percentage of all neonates develop TTN. The risk factors for TTN involve caesarian section, precipitous delivery, late preterm or early term, maternal sedation or medication, fetal distress, gestational diabetes [2]. The TTN symptoms include rapid breathing rate (> 60 breaths/minute), grunting sounds while breathing, nostril flaring, and pulling in at the ribs with breath [3-5]. Chest X-ray imaging is used to diagnose TTN. The lungs of TTN neonates display a streaked appearance and seem overinflated on X-ray. However, it is hard to distinguish TTN from other respiratory problems such as respiratory distress syndrome. Whether there is a more sensitive diagnostic biomarker for TTN has not been revealed. CD64, also known as Fcγ receptor I, is a transmembrane glycoprotein binding to monomeric IgG1, IgG3, and IgG4 with high affinity in the nanomolar range. It is constitutively expressed on monocytes and is induced on neutrophils [6]. It plays a crucial role in antibody-dependent cellular cytotoxicity and immune complex removal by macrophages [7]. Recently, neutrophil CD64 has been considered a good biomarker for a series of infections and inflammatory disorders [8-11]. In particular, it has been reported that neutrophil CD64 is a very sensitive diagnostic marker for the identification of early-onset clinical infection and pneumonia in term newborns [12] and very low birth weight infants [13]. In the current study, we tested the expression of CD64 on white blood cells together with several cellular or humoral parameters in neonates with NP and TTN, in order to select a sensitive diagnostic indicator for NP or TTN. We found that NP patients had moderately increased polymorphonuclear cells (PMNs), as well as elevated procalcitonin (PCT) and C-reactive protein (CRP) levels in the blood sera. Importantly, the expression of CD64 on PMNs was profoundly increased in NP patients but not TTN patients. Our study suggests that CD64 on PMNs could be a sensitive and reliable biomarker for NP diagnosis.

MATERIALS AND METHODS

Patients

Informed consent was obtained from each newborn infant's parents. The experimental procedures were approved by the ethical committee of Affiliated Nanhai Hospital Southern Medical University and conducted in compliance with corresponding regulations and guidelines. A total of 144 neonates, who were born in Affiliated Nanhai Hospital Southern Medical University and had signs and symptoms of anhelation, were enrolled in the study from January 2016 to September 2017. NP was diagnosed if the neonates had cough, dyspnea, and dry and wet rales of the lung, and the chest X-ray imaging showed pneumonia. TTN was diagnosed if the neonates showed respiratory distress and the lungs showed a streaked appearance and appeared overinflated on X-ray. In addition, these patients did not have perinatal asphyxia, ischemic encephalopathy, congenital disorders, hereditary metabolic disorders, and immunodeficiency.

After diagnosis, these neonates were divided into NP group and TTN group. The NP group included 66 males and 36 females aged from 0 - 1 day. The TTN group had 23 males and 19 females aged from 0 - 1 day. There was no other significant difference in the general information between the two groups. A total of 36 age-matched healthy neonates were recruited into the control group, including 18 males and 18 females.

Sample collection and analysis

No patient received antibiotics or other anti-infection therapies before the sample collection. Two milliliters of peripheral blood were collected from venipunctures and stored in a K2-EDTA blood collection tube (Thermo Fisher Scientific). WBC counting was performed on an XN-9000 hematology analyzer (Sysmex Corp). To analyze CD64 expression on neutrophils, 50 µL of whole blood was used, and the RBCs were removed by addition of 500 µL of RBC lysis buffer (Thermo Fisher Scientific) and incubation for 10 minutes at room temperature. After three washes with 5 mL of phosphate buffered saline (PBS), the cells were incubated with 2 µg/mL phycoerythrin (PE)-conjugated anti-CD64 antibody and 2 µg/mL Peridinin chlorophyll protein (Percp)-conjugated anti-CD45 antibody (both from BD Biosciences) on ice for 40 minutes in the dark. The cells were then washed twice with 1 mL of PBS and resuspended in 300 µL of PBS before being loaded on a Beckman Coulter Epics XL™ flow cytometer (Beckman Coulter Inc.). The data were analyzed using the EXPO32 software (Beckman Coulter Inc.).

To test PCT and CRP, 2 mL of peripheral blood was collected into a blood collection tube without anticoagulant (Thermo Fisher Scientific) and incubated at room temperature for 30 minutes until the blood clot was formed. The clot was removed by centrifuging at 3,000 rpm for 10 minutes in a Genius 6K-D centrifuge (Changsha Xinao instrument Co., Ltd). The sera were harvested and tested immediately or stored at -80°C before further tests. PCT was determined using the VIDAS® B.R.A.H.M.S PCT™ (Biomerieux) following the manufacturer's manual. CRP was evaluated using a Hitachi 7600-110 autoanalyzer (Hitachi).

Statistics

The data were shown as mean ± standard deviation. The unpaired *t*-test or one-way ANOVA was used to compare the mean values of each group. The difference with a p-value < 0.05 was considered to be statistically significant.

To determine the diagnostic significance of PMN CD64 index, we established the receiver operating characteris-

Table 1. Demographic characteristics of each group.

	Control	NP	TTN
n	36	102	42
Gestational age (wk)	39.7 ± 1.4	39.5 ± 0.8	39.1 ± 0.6
Male:female	18:18	66:36	23:19
Birth weight (g)	2,994 ± 79.37	3,087 ± 54.79	3,212 ± 80.08
Apgar scores			
1 minute	8 - 10	6 - 10	8 - 10
5 minutes	10 - 10	6 - 10	9 - 10
Glucose (mmol/L)	3.789 ± 0.2315	3.752 ± 0.1375	3.635 ± 0.1683
LAC	4.200 ± 0.4177	4.084 ± 0.3026	3.869 ± 0.3073
Tracheal aspirate culture	Negative	1 Haemophilus influenzae 1 Escherichia coli	Negative

tic (ROC) curves to evaluate the sensitivity and specificity of PMN CD64 index or PCT values, respectively. The ROC curve is a plot of sensitivity (%) versus 100%-specificity (%) at the values of corresponding parameters.

RESULTS

Demographic characteristics

A total of 180 newborn infants were subject to the study, including 36, 102, and 42 in the control group, NP group, and TTN group, respectively. The demographic characteristics of the three groups are summarized in Table 1. There were no remarkable differences among the groups in gestational age, birth weight, blood glucose, and other parameters listed in Table 1, although the Apgar scores at 1 and 5 minutes were slightly lower.

PMNs are increased in NP patients

To identify the cellular components that are indicative of NP and TNN, we evaluated the amounts of WBCs and platelets in the three group. As shown in Figure 1, the total WBC count was higher in the NP group (16.33 ± 0.55) in comparison to the control group (13.84 ± 0.69), but was comparable to the TNN group (14.80 ± 0.69). There was no significant difference in the lymphocyte number among the three groups (3.60 ± 0.15; 3.69 ± 0.09; 3.69 ± 0.16, respectively), while NP patients had more PMN cells (10.86 ± 0.51) than controls (8.50 ± 0.59). The PMN cell amount in TTN patients was normal (9.46 ± 0.623). Likewise, the PMN-to-lymphocytes ratio was increased in NP patients (3.09 ± 0.17). The platelet quantity seemed equal among all three groups (271 ± 9.56; 243.6 ± 5.38; 254 ± 9.06, respectively).

PCT and CRP levels are higher in NP patients

PCT is an inflammatory response indicator stimulated by bacterial products such as endotoxins and pro-inflammatory cytokines such as IL-1, IL-6, and TNFα. CRP is another inflammation indicator produced by the liver. To determine whether these two molecules are biomarkers of NP or TTN, we tested their levels in the blood. As shown in Figure 2, the PCT level was significantly higher in NP patients (1.08 ± 0.13) than that in the healthy donors (0.50 ± 0.07) and TTN patients (0.41 ± 0.08). The NP patients also presented a higher CRP concentration (2.02 ± 0.11) than healthy donors (1.52 ± 0.11), but there was no considerable difference between NP patients and TTN patients (1.61 ± 0.11).

CD64 expression on PMNs are profoundly enhanced in NP patients

It has been reported that neutrophil CD64 is a sensitive diagnostic marker for early-onset neonatal infection [12]. To ascertain whether the CD64 profile on WBCs represents a biomarker for NP or TTN, the CD64 expression was evaluated by flow cytometry. As indicated in Figure 3A, the blood lymphocytes, monocytes, and PMNs were distinguished based on CD45 expression and the side scatter intensity. The mean fluorescent intensity (MFI) of CD64 on these three cell types were then evaluated. The CD64 index was calculated by this equation: CD64 index = $(MFI_{PMN}/MFI_{Lymphocyte})/(MFI_{monocyte}/MFI_{PMN})$. As demonstrated in Figure 3B, the CD64 index of the NP group (4.93 ± 0.19) was significantly higher than that of the control group (1.34 ± 0.11) or TTN group (1.62 ± 0.12). Hence, CD64 index is a sensitive NP indicator.

PMN CD64 index is a reliable biomarker for NP diagnosis

To assess the diagnostic significance of PMN CD64 index and PCT for diagnosing NP, ROC curve analysis

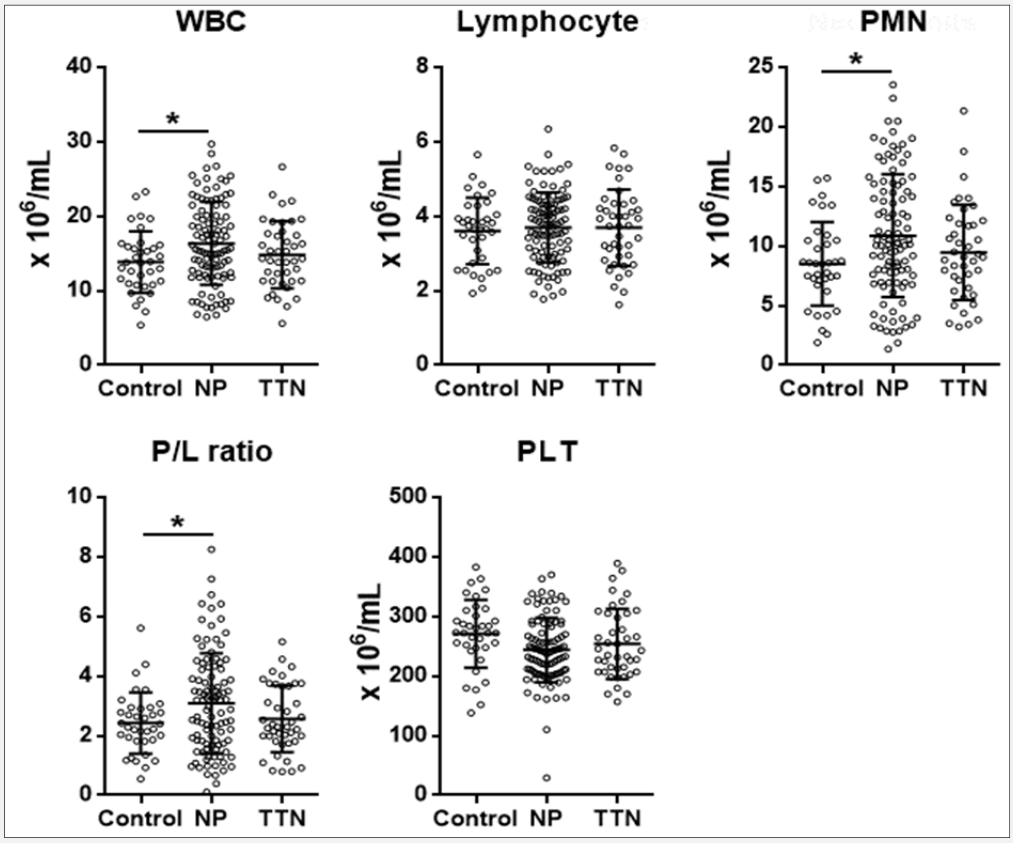

Figure 1. The values of indicated cellular parameters in the blood.

P/L ratio - the ratio between PMNs and lymphocytes, PLT - platelet, * - $p < 0.05$.

was conducted. In the ROC curves, the area under the curve (AUC) of PMN CD64 index was remarkably higher (0.917, 95% confidence interval 0.9820 - 0.997) than that of PCT (0.5515, 95% confidence interval 0.4536 - 0.6493), strongly suggesting that the PMN CD64 index is a more reliable marker for NP diagnosis. The sensitivity and specificity of PMN CD64 index was 98.04% and 91.67%, respectively, with a diagnostic threshold of 2.4. Since the AUC of PCT was just 0.5515, it is not a good indicator of NP and its sensitivity and specificity were therefore not calculated in this study.

DISCUSSION

In this study, we assessed a series of cellular or humoral parameters in neonates with NP or TTN, respectively. Interestingly, we found that the counts of WBC and PMNs were moderately up-regulated in NP patients. High blood PMN levels are often caused by bacterial infection, noninfectious inflammation or injury. In the enrolled NP patients, the culture results of tracheal aspirates were positive in only two cases. This result suggests that perhaps the bacterial titers in the tracheal aspirate were very low in the initial phase of most NP patients, although early-onset of NP is part of generalized sepsis which manifests at or within hours of birth. Many microbes can trigger NP, including gram-positive cocci, gram-negative bacilli, viruses or fungi. Hence, it is quite difficult to diagnose NP through bacterial culture or viral tests. Additionally, since high PMN amount is not a specific indicator of infection, it is not an appropriate diagnostic indicator for NP. Moreover, there was no difference in PMN counts between the NP and CRP group, further suggesting that PMN number does not necessarily point out infection or pneumonia.

Our data also indicate that PCT and CRP were mildly increased in the NP group, and PCT seems to be able to distinguish NP from TTN. Encoded by the *CALC-1*

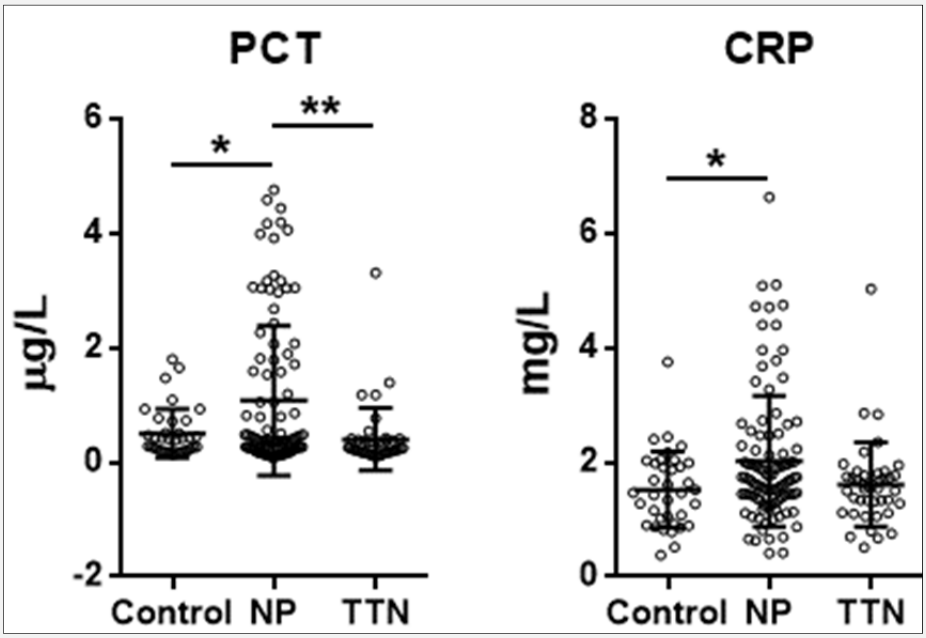

Figure 2. Serum PCT and CRP values.

*** - p < 0.05, **, p < 0.01.**

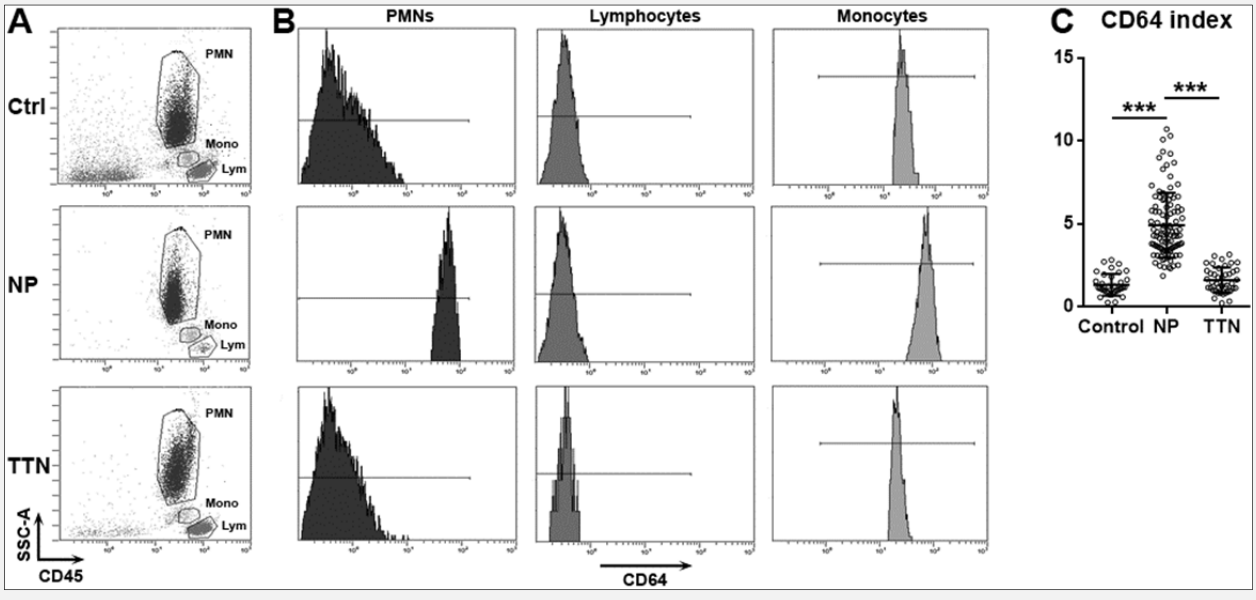

Figure 3. PMN CD64 index in each group.

(A) Representative dot plots showing PMNs, monocytes (Mono), and lymphocytes (Lym) in each group. (B) Representative.

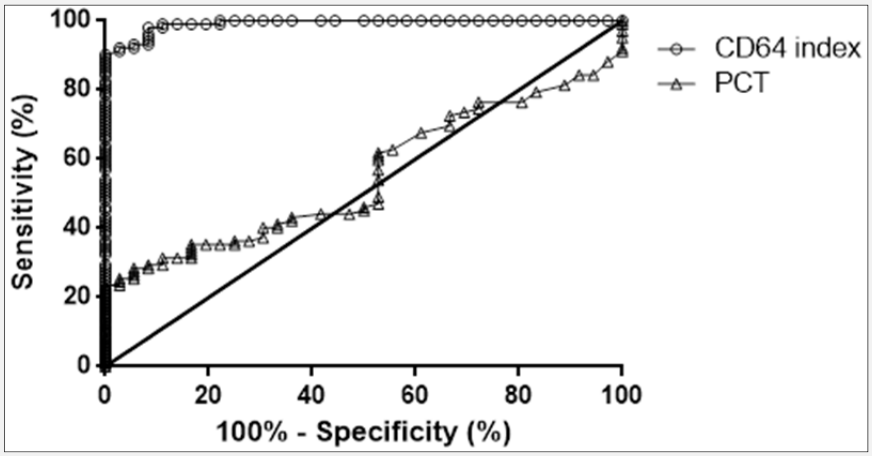

Figure 4. The ROC curves of CD64 index and PCT.

gene and consisting of 116 amino acid residues, PCT is a member of the calcitonin superfamily [14-16]. In the absence of infection, the expression of PCT is suppressed in most tissues except for the thyroid gland. Upon microbial infection, PCT expression is induced in all parenchymal tissues and distinct cell types. Its concentration rises significantly in severe systemic infections in comparison to other parameters. Even though the exact role of PCT in microbial infections remains a mystery, it is considered that the PCT test helps with the differential diagnosis of inflammatory processes. Importantly, serum PCT is a highly effective early diagnostic marker of neonatal infection [17-22]. Although CRP is also used as a marker of infection, some studies suggest that PCT is a better marker than CRP in the diagnosis of neonatal infection [17,23]. Our findings support this conclusion.

However, a thorough review of the role of PCT and CRP in the diagnosis of infection concludes that PCT has moderate accuracy in diagnosing neonatal bacterial infection, but it should be considered only within the context of other clinical parameters such as CRP, IL-6, and IL-18 and other relevant investigations [19]. In particular, PCT is not a specific biomarker for NP, and it must be combined with other tests to confirm the diagnosis of NP.

Neutrophil CD64 has been ubiquitously suggested as a reliable indicator of bacterial infection and sepsis [8,9]. Specifically, it is a very sensitive diagnostic marker for the identification of early-onset clinical infection and pneumonia in term newborns [12] and very low birth weight infants [13]. Interestingly, it could possibly distinguish acute inflammatory autoimmune disease from systemic infections [10]. Our data indicate that the PMN (mostly neutrophils) CD64 index was remarkably increased in NP patients as compared with healthy neonates and TTN neonates, suggesting that neutrophil CD64 is a sensitive marker for NP even when the bacterial titer is very low. Hence, it would be beneficial to apply the flow cytometry assay on neutrophil CD64 expression to screen possible bacterial infections in infants. Moreover, combining the neutrophil CD64 test with respiratory distress symptoms could be an easy way to tell NP and subsequently facilitate quick therapies.

Although the up-regulation of neutrophil CD64 in NP patients was noteworthy, it is still unclear what causes this up-regulation and the role of CD64 in neutrophil function. CD64 is also known as Fc gamma RI. On the surface of macrophages, CD64 binds IgG with high affinity and mediates antibody-dependent cellular cytotoxicity and immune complex clearance during early immune responses. Knockout mice documented a critical role for FcγRI in models of *Bordetella pertussis* clearance and Arthus reaction [24-26]. CD64 might exert similar effects on neutrophils during infection. Indeed, former research demonstrates that CD64 is involved in the phagocytosis and killing of *Candida albicans* (a fungus) by neutrophils [27]. It is therefore plausible to speculate that neutrophil CD64 plays an important role in the removal of pathogenic bacteria in NP neonates.

CONCLUSION

Collectively, this study suggests that in comparison with the tested parameters, CD64 expression on PMNs could be a sensitive biomarker for NP diagnosis. However, no good biomarker has been found for TTN detec-

tion. Future investigations are needed to explore other diagnostic indicators in NP and TTN neonates.

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Declaration of Interest:
There is no conflict of interest.

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