

Abnormal Expression of Peripheral Blood Leukocyte Surface Markers in Retinopathy of Prematurity Patients

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Objective: Our study compares immune cell profiles in preterm infants with and without severe ROP, identifying risk factors for its development to explore both immunological aspects and determinants of ROP in preterm infants.

Methods: Infants born between January 2023 to December 2023 at the Children's Hospital of Shanxi were enrolled in this study according to the inclusion criteria. Patients were divided into a test group or a control group based on the need for Type 1 ROP treatment. Baseline data for both groups were compared. Neutrophil, lymphocyte, and monocyte subsets in the peripheral blood were analyzed using immunophenotypic analysis via multicolor flow cytometry. This method allowed for the quantification of specific cell subset proportions.

Results: A total of 2,110 preterm infants were screened for inclusion in the study. Multivariate logistic regression analysis identified gestational age below 28 weeks, birth weight less than 1,000 g, and neonatal sepsis as independent risk factors for severe ROP. Furthermore, flow cytometry analysis was performed on blood samples from 45 preterm patients. Comparative analysis revealed that the test group had a lower percentage of neutrophils and higher expression of cluster of differentiation 81 (CD81) compared to the control group. Additionally, the test group showed a higher percentage of lymphocytes and a greater proportion of Th17 cells than the control group.

Conclusion: Preterm gestational age, low birth weight, and neonatal sepsis increase severe ROP risk. Elevated CD81 and Th17 levels suggest inflammation linked to neutrophils and lymphocytes.

Keywords: flow cytometry, inflammatory response, lymphocytes, neutrophils, retinopathy of prematurity

Background

As advancements in neonatal care improve survival rates of preterm infants, retinopathy of prematurity (ROP) has become a leading cause of blindness in neonates worldwide. In China, the incidence of ROP has reached 17.8%.¹ The occurrence of ROP places significant economic burden on both families and society as a whole.²

The underlying mechanisms driving the onset of ROP have been increasingly elucidated, with growing attention focused on the role of immune responses and inflammation in its initiation and progression.³ Over the past few decades, multiple studies have investigated the impact of immune and inflammatory responses on ROP development. Accumulating evidence suggests that inflammation plays a key role in regulating retinal neovascularization, thereby contributing to the pathogenesis of ROP.⁴ Neutrophils, the predominant white blood cells in human circulation, are critical in immune defense against microbial pathogens, including bacteria and fungi. They are also central to the

initiation and progression of inflammatory responses.⁵ Notably, studies have shown that on the first day after birth, neonates with ROP exhibit a lower white blood cell count compared to the control group. Additionally, inflammatory markers such as the neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), and platelet-to-lymphocyte ratio (PLR) have been identified as potential biomarkers for predicting outcomes in various diseases. Hu et al reported a negative correlation between ROP and lymphocyte count, further noting an association between the LMR and the development of ROP.⁶

Despite these findings, there remains a paucity of research comprehensively investigating the role of neonatal neutrophils in the onset and progression of ROP. The impact of neutrophils can be assessed by examining the expression of surface markers such as cluster of differentiation 11b (CD 11b), CD45, CD62L, CD66b, CD74, CD81, and CD127, which regulate leukocyte mobilization, adhesion, and activation.^{7,8} Therefore, further research is needed to better understand the role of neonatal immune and inflammatory responses in the progression of ROP.

⁹ The pathogenesis of ROP is multifactorial, involving the complex interplay of factors such as oxygen levels, angiogenic growth factors, and inflammatory mediators.^{10,11} Inflammation is considered to play a crucial role in the development of ROP, with studies showing elevated levels of pro-inflammatory cytokines in the vitreous and serum of infants with ROP.^{12–14} However, the precise role of inflammation in the pathogenesis of ROP, as well as its relationship to disease severity, remains unclear.

Methods

Study Participants

A prospective cohort study was conducted involving neonates admitted to the Neonatology Department of Children's Hospital of Shanxi between January 2023 and December 2023, encompassing those with a gestational age of ≤ 32 weeks and a birth weight of $\leq 2,000$ g.¹⁵ approved by the Ethics Committee of Ethics Committee of the Children's Hospital of Shanxi and Women Health Center of Shanxi Affiliated to Shanxi Medical University (Approval Number: IRB-KYYN-2023-013). Eye fundus screening, treatment, and subsequent follow-up were performed according to the criteria outlined in *the International Classification of Retinopathy of Prematurity (3rd edition)*.¹⁶ Exclusion criteria included patients with congenital cataracts, familial exudative vitreoretinopathy, primary vitreous hyperplasia, ocular trauma, or those whose parents or legal guardians declined participation. All eligible neonates underwent Retcam[®] III screening and subsequent follow-up assessments. Patients in the test group were diagnosed with Type 1 ROP, which is classified into four distinct classifications: (1) Type 1 ROP in zone 1, any stage with plus disease; (2) Type 1 ROP in zone 1, stage 3 without plus disease; (3) Type 1 ROP in zone 2, stage 2; and (4) Type 1 ROP in zone 2, stage 3. Concurrently, neonates treated in the Neonatology Department of the Children's Hospital of Shanxi during the same period, but found not to have ROP based on eye fundus screening, were randomly selected to form the control group.

Venous blood samples were collected from both the test and control groups before the administration of intravitreal ranibizumab injections in both eyes and before discharge from the neonatal intensive care unit. In addition to routine blood tests, venous blood was drawn into heparin anticoagulant tubes. A 1 mL whole blood sample was collected and transported to the laboratory within two hours, kept in an icebox for preservation during transit. (Refer to [Figure 1](#) for the flowchart illustration).

Laboratory Analysis

Immunophenotyping using flow cytometry was conducted according to a defined protocol.¹¹ According to this protocol, initially, 50 μ L of whole blood, anticoagulated with ethylenediaminetetraacetic acid (EDTA), underwent erythrocyte lysis, followed by washing and separation to obtain peripheral blood immune cells. These isolated cells were then incubated with fluorescent-conjugated monoclonal antibodies (refer to [Table 1](#)) in a dark environment at room temperature for 30 minutes. All reagents were purchased from Biolegend[®] (San Diego, CA) and used in accordance with the manufacturer's instructions. All samples were processed within three hours after collection.

Upon completion of sample preparation, the specimens were promptly analyzed using CellQuest[™] Pro software (BD Biosciences, San Diego, USA) interfaced with a FACSCalibur flow cytometer. Approximately 50,000 events were

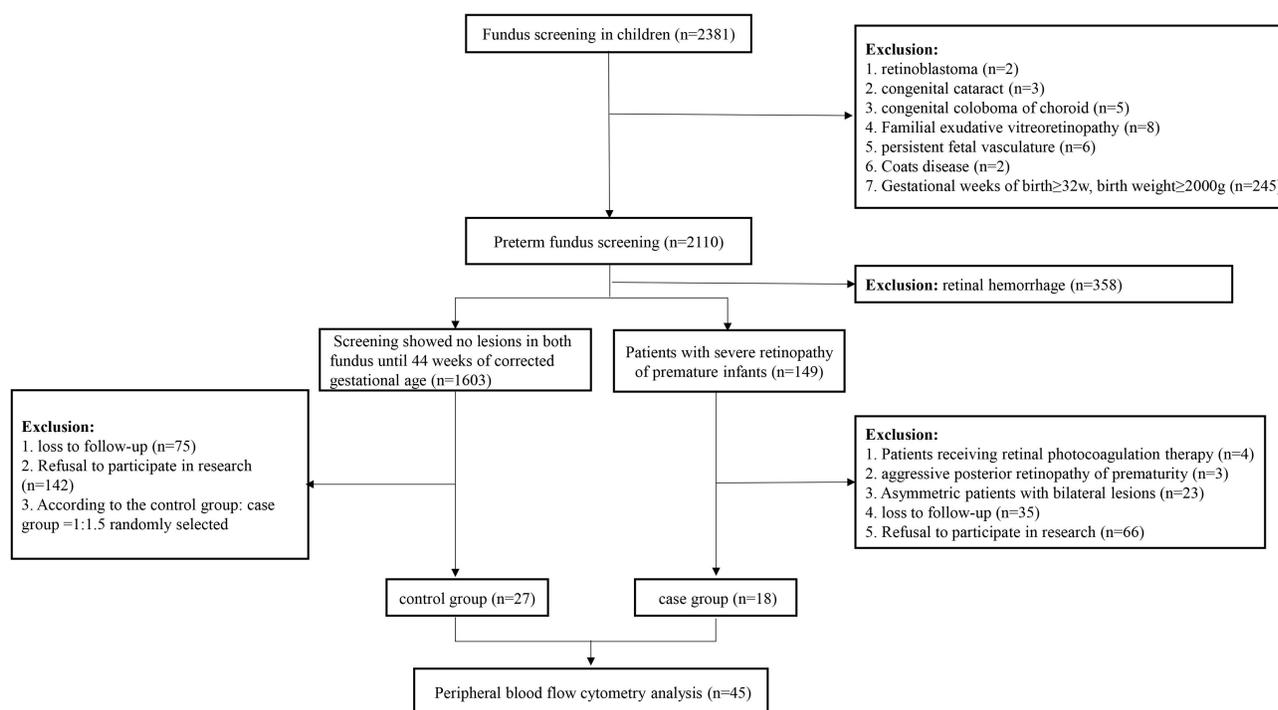


Figure 1 Flowchart depicting the screening process of study participants.

recorded from each sample. Reproducibility of fluorescence intensity was ensured through calibration and routine quality control procedures. Subsequent data analysis was performed using FlowJo software (TreeStar, Ashland, OR). Regarding the gating strategy, neutrophils were selected based on their forward scatter (FSC) and side scatter (SSC) distributions, with debris excluded. The percentage of neutrophils and the mean fluorescence intensity (MFI) of the markers under investigation were recorded (Tables 2–4).

Table 1 Maternal and Neonatal Baseline Characteristics (n=301)

	NC (152)	ROP (149)	t	P
Gestational age (weeks)	29.47±2.00	27.19±1.36	3.176	0.004 ^a
Birth weight (g)	1243.75±342.4	913.13±110.74	3.512	0.002 ^a
sex				P
male	56.58% (86)	51.01% (76)	0.122	0.982
female	43.42% (66)	48.99% (73)		
Neonatal Apgar score				
Apgar I	9(8/9)	8(7/8)	1.266	0.531
Apgar5	10(9/10)	9(9/9)	4.613	0.061
Type of delivery				
Vaginal	31.58% (48)	74.50% (111)	4.112	0.053
C-section	68.42% (104)	25.50% (38)		
Neonates with systemic disease				
Neonatal purulent meningitis	19.08% (29)	11.41% (17)	0.362	0.547
Neonatal sepsis	53.33% (81)	9.01% (13)	4.350	0.037 ^a
Neonatal pneumonia	6.25% (9)	29.87% (42)	2.666	0.102

(Continued)

Table 1 (Continued).

	NC (152)	ROP (149)	t	P
Maternal complications during pregnancy				
Hypertensive disorder complicating pregnancy	24.48% (37)	9.78% (14)	0.891	0.345
Gestational diabetes mellitus	25.02% (38)	19.87% (30)	0.087	0.768
hypothyroidism	18.75% (28)	20.01% (30)	0.006	0.937
Antiphospholipid antibody syndrome	6.25% (9)	7.76% (12)	1.140	0.286
Perinatal use of antibiotics	49.87% (76)	29.62% (44)	1.008	0.315
Prenatal use of dexamethasone	62.5% (95)	59.74% (89)	0.016	0.899

Notes: a: Statistically significant result. NC: Normal Control. t-test: Student's t-test. P-value: A P-value of P < 0.05 indicates a statistically significant difference. Apgar score: The parentheses in the cell represent the median (25th percentile/75th percentile).

Table 2 Flow Cytometry Panels of Lymphocytes

Fluorochromes	Antibody	Dose
FITC	CD45	1ul
BV650	CD19	3ul
BV711	CD3	1ul
PE-CY5	CD4	1ul
AF700	CD8	1ul
PE	CXCR3	1ul
PE-CY7	CCR6	1ul
BV786	CD25	1ul
AF647	CD127	1ul
Percp-cy5.5	CD45Ra	1ul
BV421	CCR7	1ul
BV605	CD38	1ul
APC-H7	HLA-DR	1ul
BV510	CD138	3ul

Table 3 Flow Cytometry Panels of Lymphoid Cells

Fluorochromes	Antibody	Dose
FITC	CD45	1ul
BV605	CD3	1ul
BV510	CD11b	1ul
PE	CD11c	1ul
BV421	CD66b	1ul
PE-CY5	CD14	1ul
PE-CY7	CD86	1ul
BV711	CD206	3ul
AF647	CD68	3ul
pe-DZ594	TCR $\gamma\delta$	3ul
APC-R700	CD56	1ul
BV786	CD16	1ul
Percy-cy5.5	CD123	1ul
APC-H7	HLA-DR	1ul
BV650	CD19	3ul

Table 4 Flow Cytometry Panels of Neutrophils

Fluorochromes	Antibody	Dose
AF700	CD45	1ul
BV650	CD11b	1ul
Percp-cy5.5	CD66b	3ul
PE-CY7	CD81	1ul
BV605	CD62L	1ul
APC	CD74	1ul
PE	CD54	1ul
APC-CY7	CD127	1ul

Flow Cytometry

Fresh anticoagulated blood was collected and centrifuged at 400g for 5 minutes at 4°C. The supernatant is discarded, and red blood cells were lysed with a lysis buffer for 1–2 minutes. After repeated centrifugation and washing steps, the cell pellet was resuspended in PBS. The cell suspension was divided into three tubes (lymphoid, myeloid, and neutrophil lineages), with 200 µL per tube. Antibodies were added at a 1:200 dilution, mixed, and incubated at 4°C for 30 minutes. After incubation, the cells were washed and resuspended in PBS for flow cytometry analysis. Data were acquired using a FACSCalibur flow cytometer with BD FACSDiva software, and at least 10,000–50,000 events were recorded per sample. Data analysis was performed using FlowJo software, focusing on identifying specific cell subpopulations and measuring mean fluorescence intensity (MFI).

Statistical Analysis

The baseline data and blood routine analyses of the patients were assessed using SPSS 29.0 statistical software (IBM co, United States). The enumeration data were represented as the mean ± standard deviation. Normality of continuous variables was first evaluated using the Shapiro–Wilk test, with $P > 0.05$ indicating a normal distribution. Normally distributed continuous variables were compared between the two groups using a one-way analysis of variance (ANOVA) based on the distribution. Subsequent pairwise comparisons were conducted using the least significant difference (LSD) method. For non-normally distributed continuous variables, logarithmic transformation was employed to convert them into normally distributed variables before analysis. Measurement data were presented as percentages, with inter-group comparisons conducted using the chi-squared test. Factor analysis was conducted using logistic regression models.

The flow cytometry data are presented as the mean ± standard error of the mean (SEM). One-tailed t-tests were used to assess the significance of the differences between the two groups. These tests were conducted using the GraphPad Prism software (GraphPad, San Diego, CA). Statistical significance was defined as a P -value less than 0.05 ($P < 0.05$).

Ethical Aspects

This research received approval from the Ethics Committee of the Children’s Hospital of Shanxi (Women Health Center of Shanxi), as indicated by the ethics approval number IRB-KYYN-2023-013. It was implemented in compliance with the principles outlined in the *Declaration of Helsinki* and adhered to the prevailing laws in China. Before participation, the father, mother, or legal guardian provided written informed consent, on behalf of the participants. Blood samples were collected in EDTA tubes by medical staff for subsequent analysis using discarded samples. No dedicated blood sampling, including venipuncture or arterial puncture, was conducted exclusively for this study.

Results

General Data of the Patients

In this investigation, a total of 2,110 preterm infants were screened, with an average gestational age (GA) of 28 weeks (range: 26–32 weeks) and an average birth weight (BW) of 1,107 grams (range: 600–1,950 grams). Among these cases, 1,039 (49.24%) were female and 1,071 (50.77%) were male. Within this cohort, 358 (16.97%), ROP cases were identified through screening. Specifically, screening identified 1,603 (75.97%) cases of patients with no lesions in both eyes with a corrected gestational age of 44 weeks and 149 (7.06%) cases of patients diagnosed with Type 1 ROP requiring treatment (refer to [Figure 1](#)).

The following variables were collected prospectively: gender, birth weight, gestational age, mode of delivery, prenatal administration of antibiotics, dexamethasone, Apgar score, and accompanying neonatal sepsis, pneumonia, and purulent meningitis, as well as maternal comorbidities during pregnancy (namely, hypertension, diabetes mellitus, hypothyroidism, and antiphospholipid antibody syndrome).

Multivariate Logistic Regression

The control group and the test group demonstrated statistical differences in birth gestational age, birth weight, and the occurrence of neonatal sepsis, whereas no statistical differences were noted in the remaining factors. [Table 5](#) presents a further analysis of relevant risk factors.

Additionally, statistical differences were detected in the absolute values of neutrophils and lymphocytes between the control and test groups, as evidenced by the statistical analysis of the blood routine indicators presented in [Table 6](#).

Furthermore, the Neutrophil-Lymphocyte Ratio (NLR) was calculated and revealed a significant difference between the groups. The ROC curve analysis for NLR, as shown in [Figure 2](#), demonstrated an Area Under the Curve (AUC) of 0.759, indicating moderate discriminatory ability. The maximum Youden index (0.552) corresponded to an NLR cutoff value of 0.5650, which could be used as a diagnostic threshold for Retinopathy of Prematurity (ROP). At this NLR cutoff, the sensitivity was 65.2%, and the specificity was 90%, suggesting its potential as a clinically useful marker for ROP diagnosis.

Table 5 Logistic Regression Analysis of Related Factors Associated with ROP

Factors	Regressive Coefficient B	Standard Error	Wald	P value	OR	95% CI
Gestational age < 28w	-0.159	0.134	2.93	0.002	1.612	(1.187,2.246)
Birth weight <1,000g	-0.102	0.019	11.02	<0.01	1.538	(5.727,12.976)
sex	1.238	0.342	7.22	0.36	2.47	(1.312–6.143)
Neonatal Apgar score	4.652	0.234	6.38	0.45	1.96	(1.161–4.702)
Type of delivery	2.376	1.231	2.12	0.34	1.32	(1.011–4.964)
Neonates with systemic disease						
Neonatal purulent meningitis	2.487	0.259	8.73	0.52	3.71	(1.236–4.451)
Neonatal sepsis	-0.287	0.178	2.09	0.04	1.826	(1.019,2.302)
Neonatal pneumonia	-9.231	3.231		0.24	3.05	(0.921–2.272)
Maternal complications during pregnancy						
Hypertensive disorder complicating pregnancy	-0.452	0.891	7.21	0.61	1.67	(0.857–2.413)
Gestational diabetes mellitus	-3.235	3.579	5.32	0.78	1.12	(1.361–6.152)
hypothyroidism	4.341	3.245	6.21	0.82	1.46	(2.172–7.423)
Antiphospholipid antibody syndrome	7.239	4.782	7.23	0.27	2.13	(1.317–6.146)
Perinatal use of antibiotics	5.237	5.823	8.29	0.24	1.04	(1.937–10.289)
Constant	-0.182	0.606	0.09	0.036	0.013	

Table 6 Comparison of Blood Routine Between the Control and Test Groups (n = 376)

Indicator	Mean \pm SD		F	T value	P value
	NC	ROP			
White blood cell count	10.28 \pm 3.46	9.86 \pm 2.84	0.562	0.338	0.737
Absolute neutrophil count	3.8 \pm 2.66	2.23 \pm 0.9	3.465	1.799	0.041
Absolute lymphocyte count	4.39 \pm 1.69	6.13 \pm 3.14	0.806	0.376	0.043
Absolute monocyte count	1.32 \pm 0.4	1.23 \pm 0.43	0.392	1.207	0.237
Absolute eosinophil count	0.47 \pm 0.24	0.40 \pm 0.22	0.184	0.816	0.421
Absolute basophil count	0.03 \pm 0.03	0.03 \pm 0.03	0.02	0.036	0.972

Notes: Units of cell counts: $10^9/L$.

Flow Cytometry Test results

Flow cytometry analysis was performed on blood samples obtained from 45 patients. The test group exhibited a lower percentage of neutrophils in comparison to the control group, with a significantly larger CD81+PMN (polymorphonuclear neutrophils) subset in the test group than in the control group, as illustrated in Figure 3. Furthermore, the test group exhibited an elevated percentage of CD4+T lymphocytes in comparison to the control group. Remarkably, the test group displayed significantly larger Th1 and Th17 cell subsets, alongside notably smaller Treg subsets compared to the control group, as depicted in Figure 4.

Discussion

This study identifies preterm gestational age, low birth weight, and neonatal sepsis as significant independent risk factors for retinopathy of prematurity (ROP). Specifically, infants born with a gestational age <28 weeks or a birth weight <1,000 g face a heightened risk of developing ROP requiring treatment, with odds ratios of 1.612 and 1.538, respectively.

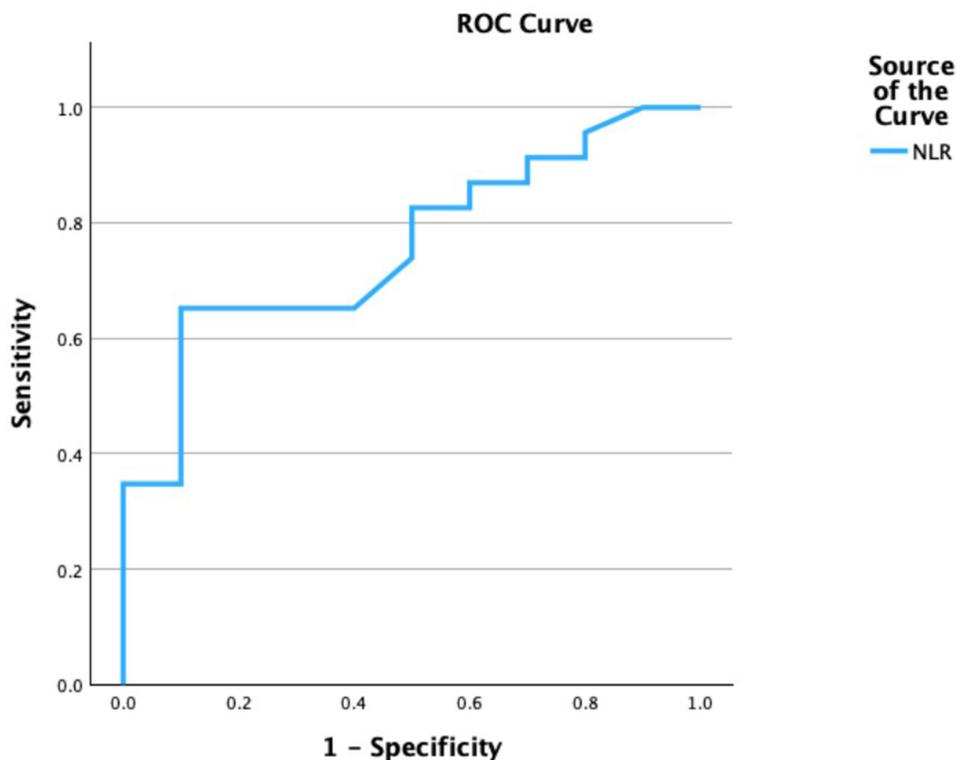
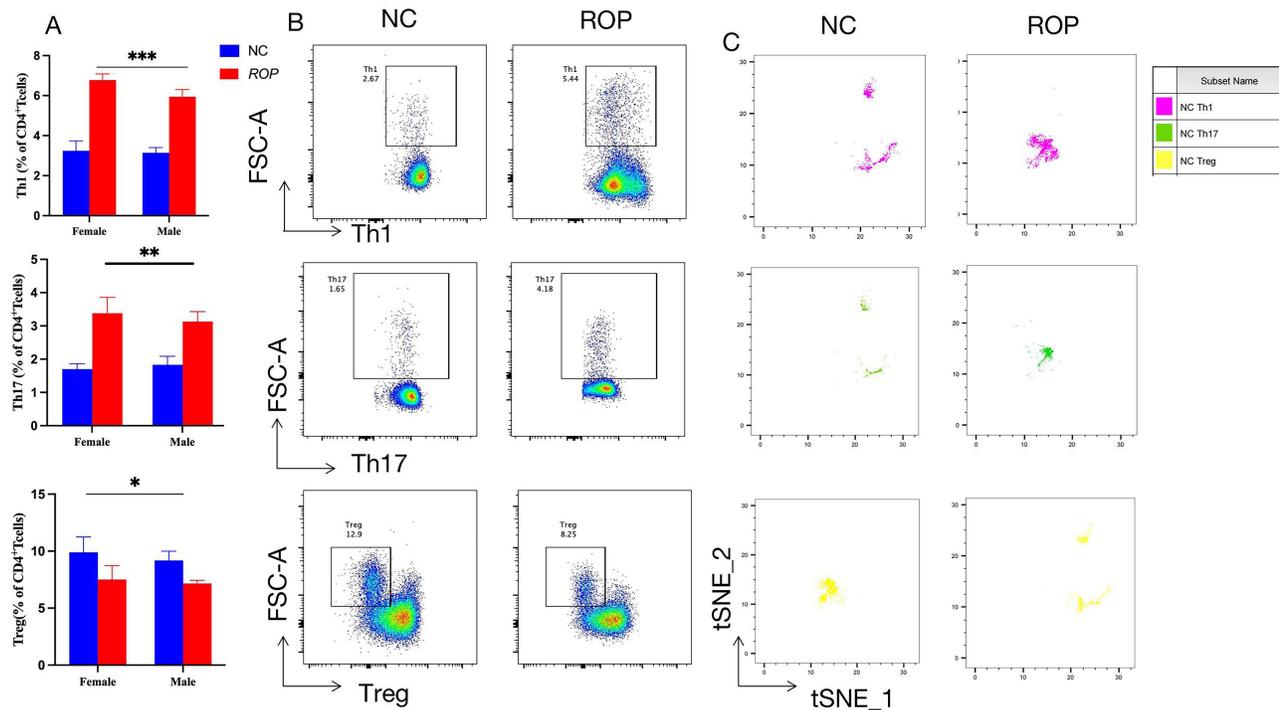
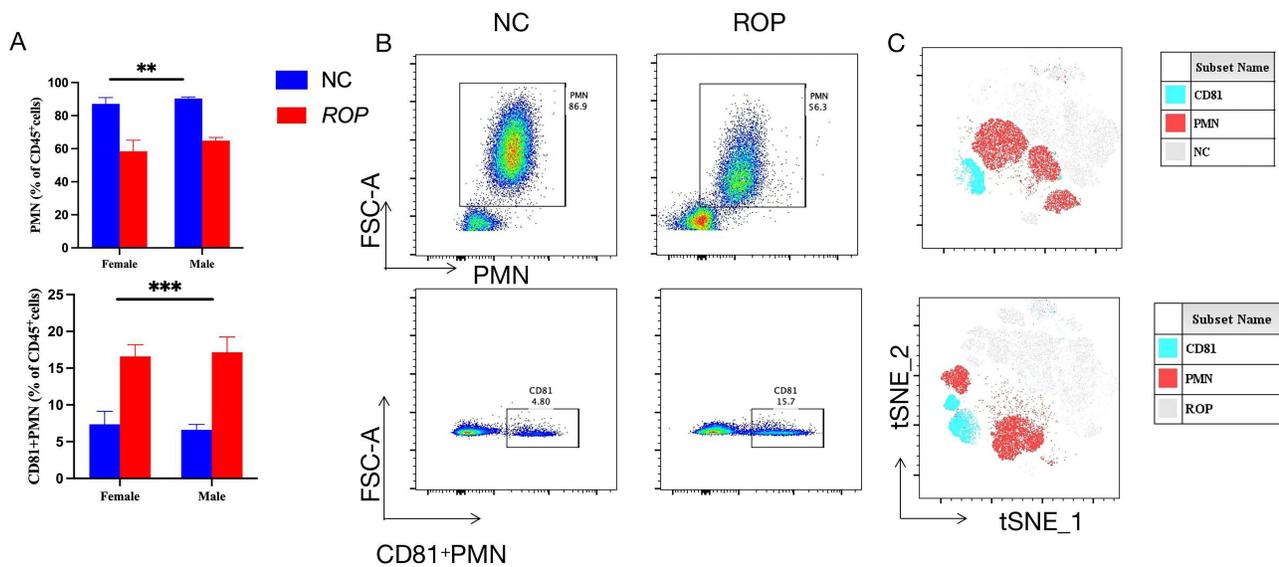


Figure 2 ROC curve analysis of neutrophil-lymphocyte ratio (NLR) for diagnosis of retinopathy of prematurity (ROP).



Furthermore, neonatal sepsis increases the risk of severe ROP by 1.826-fold. These findings align with previous studies and underscore the critical role of early neonatal factors in ROP development.¹⁷⁻¹⁹

Our immunohistochemical analysis highlights the expression of key immune markers associated with ROP. Notably, the proportion of CD11b+CD66b+ neutrophils was significantly lower in the ROP group compared to controls, suggesting a potential congenital immune deficiency in these infants. Conversely, CD4+ Th lymphocytes were more

abundant in the ROP group, pointing to a potential shift in the immune response that may contribute to ROP pathogenesis. These findings are consistent with previous studies on the role of immune cells in ROP progression.^{6,20,21}

Inflammation appears to play a pivotal role in ROP development, with neonatal sepsis and systemic inflammation influencing retinal neovascularization. Previous studies have also linked inflammatory markers, such as the neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR), to ROP risk, although findings have been inconsistent. Our study extends this work by using multi-parameter flow cytometry to provide a detailed profile of peripheral blood immune cells. We found that the CD81+ neutrophil subset was elevated in the ROP group, which may indicate a role in modulating both innate and adaptive immune responses. The balance between neutrophils and lymphocytes, as reflected in the NLR, may be critical in ROP pathogenesis.^{6,20,22,23}

Furthermore, the interaction between inflammatory cytokines, particularly IL-17, and retinal angiogenesis in preterm infants is a key area for future investigation. The dysregulated immune response, including alterations in T-cell differentiation and immune synapse formation, may contribute to the pathological vascular changes seen in ROP.^{24–26} This is consistent with findings from Borțea et al, who observed elevated inflammatory markers in infants with ROP, suggesting that systemic inflammation may drive retinal neovascularization.²⁰ Our study's findings regarding the elevated CD81+ neutrophil subset further support the hypothesis that immune dysregulation contributes to ROP development.

This study offers several advantages, including its comprehensive analysis of immune cell dynamics in preterm infants with ROP. Key strengths include extensive hospitalization data, in-depth multi-parameter flow cytometry analysis of immune cell subsets, and a detailed examination of peripheral blood immune cells. The findings align with the outcomes of Reference 6, but expand on them by investigating the heterogeneity of peripheral blood immune cell subsets in ROP patients, which was not explored in the clinical trial.

However, the study has limitations. The primary constraint is the use of discarded blood samples from EDTA tubes, which were analyzed upon request, potentially affecting immune cell activity. Additionally, the complex immune dysfunction in ROP involves intricate immune cell interactions, cytokines, and transcription factors, necessitating further investigation. Future studies should focus on the influence of peripheral blood immune cells on the retinal immune environment, including retinal cells and blood vessels, as well as the role of CD81+PMN subgroups in modulating Th17 cell responses to oxidative stress.

Another limitation is the reliance on peripheral blood samples to infer ocular immune states, which may not accurately reflect local immune processes in the eye. The ocular microenvironment and blood-retinal barrier may have distinct immune responses not captured by peripheral blood analysis. Future research incorporating ocular-specific samples and multi-omics data is essential to better understand the immunopathogenesis of ROP and identify potential therapeutic targets and diagnostic biomarkers.

Conclusion

This study highlights the pivotal role of inflammation in the development and progression of retinopathy of prematurity in preterm infants. Elevated levels of inflammatory markers are associated with an increased risk of ROP development and progression, with infants with ROP demonstrating pronounced inflammatory profiles compared to those without ROP. Our results indicate that a gestational age of less than 28 weeks, a birth weight of less than 1000g, and the co-occurrence of neonatal sepsis are significant risk factors for the onset and progression of ROP. Moreover, increased peripheral blood neutrophils and lymphocytes are significantly correlated with an elevated risk of ROP, underscoring their potential as biomarkers for ROP risk prediction. Overall, this study emphasizes the critical need for early and ongoing monitoring of inflammatory markers in preterm infants and suggests the potential of inflammation-targeted therapeutic strategies to mitigate the risk and severity of ROP in this vulnerable population. Future research should focus on validating these findings in larger multicenter cohorts and exploring the mechanistic pathways that link inflammation to ROP, in order to further refine our understanding and facilitate the development of targeted interventional measures.

Abbreviations

ROP, retinopathy of prematurity; BW, birth weight; GA, gestational age; PMN, polymorphonuclear neutrophils; NC, normal control.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

This study was conducted with approval from the Ethics Committee of Ethics Committee of the Children's Hospital of Shanxi and Women Health Center of Shanxi Affiliated to Shanxi Medical University (Approval Number: IRB-KYYN-2023-013). This study was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from all patients' legal guardians.

Consent for Publication

All patients' guardians signed a document of informed consent.

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Disclosure

The authors declare that they have no competing interests for this work.

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