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IL-27 as a potential biomarker for distinguishing between necrotising enterocolitis and highly suspected early-onset food protein-induced enterocolitis syndrome with abdominal gas signs

Yuhong Qi[a], Chan Liu[b], Xin Zhong[b], Xueling Ma[b], Jie Zhou[c], Yuan Shi[b], Yibing Yin[a], Xuemei Zhang[a], Yu He[b,*], Wenchun Xu[a,*]

[a] Key Laboratory of Clinical Laboratory Diagnostics of Ministry of Education, Faculty of Laboratory Medicine, Chongqing Medical University, China
[b] Department of Neonatology, National Clinical Research Centre for Child Health and Disorders, Ministry of Education Key Laboratory of Child Development and Disorders, Chongqing Key Laboratory of Pediatrics, China International Science and Technology Cooperation Base of Child Development and Critical Disorders, Children's Hospital of Chongqing Medical University, Chongqing, China
[c] Department of Clinical Laboratory, Ministry of Education Key Laboratory of Child Development and Disorders, National Clinical Research Centre for Child Health and Disorders, China International Science and Technology Cooperation base of Child development and Critical Disorders, Chongqing Key Laboratory of Pediatrics, Children's Hospital of Chongqing Medical University, Chongqing, China

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ABSTRACT

Background: The initial clinical manifestations and abdominal imaging findings of neonates with necrotising enterocolitis (NEC) and food protein-induced enterocolitis syndrome (FPIES) are sometimes similar; however, their prognosis and therapies are different. We aimed to evaluate the utility of interleukin (IL)-27 as a differentiation marker between NEC and highly suspected early onset (HSEO)-FPIES.
Methods: All samples used in this study were obtained from the neonatal diagnosis centre of Children's Hospital of Chongqing Medical University. In the case-control study, neonates with NEC (n = 13), HSEO-FPIES (n = 9), and jaundice (control, n = 8) were enroled to determine the serum IL-27 levels using commercial enzyme-linked immunosorbent assay (ELISA) kits. In the validation cohort study, the NEC (n = 87), HSEO-FPIES (n = 62), and jaundice (control, n = 54) groups were included to analyse the diagnostic efficiency of IL-27 for discriminating between NEC and HSEO-FPIES using a receiver operating characteristic (ROC) curve.
Findings: In the case-control study, IL-27 levels were higher in the NEC group than in the HSEO-FPIES group (p = 0·005). In the cohort study, the area under the ROC curve (AUC) of IL-27 for differentiating NEC from HSEO-FPIES was 0·878, which was higher than the AUCs of IL-6 (0·761), C-reactive protein (0·800), white blood cell count (0·637), neutrophils (0·765), lymphocytes (0·782), neutrophil to lymphocyte ratio (0·781), and platelet count (0·729).
Interpretation: Serum IL-27 is a novel biomarker that may potentially discriminate NEC from HSEO-FPIES in neonates.

1. Introduction

Necrotising enterocolitis (NEC) and food protein-induced enterocolitis syndrome (FPIES) are common gastrointestinal disorders in neonates. NEC is a devastating intestinal disease and thus has become the primary focus of many studies [1], and its incidence can be as high as 13% in neonates born at or less than 33 weeks of gestation or whose birth weight is less than 2500 g [2]. The mortality of NEC ranges from 20 to 30% and can reach as high as 50% in surgical NEC cases [3]. The treatment of NEC is complicated and expensive, and the current diagnostic standard is the modified Bell's staging criteria, which are primarily based on clinical manifestations and radiologic features [3].

FPIES is a non-IgE-mediated gastrointestinal food hypersensitivity [4]. The most common food triggers for FPIES are cow's milk and soy proteins [5]. There is a lack of large population-based FPIES studies [4]. Unrecognised FPIES results in repeated attacks and chronic symptoms with poor weight gain and failure to thrive [6,7]. Oral food challenges are the gold standard for diagnosis of FPIES, but they are seldom performed in the clinic [5]. Thus, the current diagnostic criteria for FPIES are primarily based on medical history, clinical manifestations, and symptomatic relief after dietary avoidance [7,8].

* Corresponding authors.
E-mail addresses: heyu@hospital.cqmu.edu.cn (Y. He), xuwen@cqmu.edu.cn (W. Xu).

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Research in context

Evidence before this study

It is difficult to differentiate necrotising enterocolitis (NEC) and food protein-induced enterocolitis syndrome (FPIES) with abdominal gas signs in neonates due to their similar clinical manifestations and abdominal imaging findings in the early stage. However, their prognosis and therapies vary greatly. Many studies have assessed biomarkers for the diagnosis of NEC; however, none of them have confirmed whether these biomarkers can effectively differentiate FPIES from NEC, although early differentiation is much needed in the clinic. IL-27 expression can be upregulated by toll-like receptor 4 signalling, which plays a critical role in the pathogenesis of NEC. Serum levels of IL-27 were significantly increased in the experimental models of inflammatory bowel disease, but they were decreased in patients with allergic diseases. It is not known whether IL-27 can be a potential biomarker to discriminate NEC from highly suspected early onset (HSEO)-FPIES.

Added value of this study

IL-27 levels were significantly higher in the NEC group than in the HSEO-FPIES group. Also, IL-27 was found to be a potential biomarker to differentiate NEC from HSEO-FPIES with an area under the receiver operating characteristic curve (AUC) of 0·878, sensitivity of 88.37%, and specificity of 75.81%. In addition, extremely elevated IL-27 levels were correlated with the severity of NEC.

Implications of all the available evidence

FPIES is a relatively newly identified entity during the neonatal period, and in some cases, early differentiation between HSEO-FPIES and NEC is difficult. To our knowledge, this is the first study to explore a biomarker that can discriminate between NEC and HSEO-FPIES in neonates in a relatively large study population, and the findings suggest that IL-27 may potentially help the neonatologists in the decision-making process.

In the clinic, FPIES with pneumatosis intestinalis, portal venous gas, and pneumoperitoneum on radiographs or ultrasound, which were defined as abdominal gas signs in our study, sometimes mimics NEC due to the similarities of symptoms and abdominal imaging findings, making it difficult to distinguish NEC from FPIES in neonates [5,8–10]. Firstly, the clinical symptoms of NEC and FPIES, such as vomiting, abdominal distension, diarrhoea, bloody stool, feeding difficulties, sleepiness, apnoea, and shock, might be similar [3,8]. Secondly, in both disorders, patients show intestinal dilatation, pneumatosis intestinalis, and portal venous gas on radiographs or ultrasound [5,7]. However, the prognosis and therapies for NEC and FPIES are vastly different [5,7]. NEC cases tend to experience rapid escalation of symptoms, much poorer prognosis, and need a much longer duration of antibiotics and fasting, whereas FPIES requires only dietary modification [5,7]. In most cases, the neonatologists can distinguish between the two conditions in the late stage of the disease after reviewing the disease process [5]. However, in the early stage, differential diagnosis is difficult. Few studies have explored the laboratory indicators to discern NEC from FPIES. The levels of routine laboratory indicators, such as platelet (PLT) count, white blood cell (WBC) count, and C-reactive protein (CRP) levels, have shown different trends in NEC and FPIES; however, they could not effectively discriminate between the two disorders [5,7,11–13]. Thus, there is an urgent clinical need to identify a new biomarker to differentiate between these two disorders, which could greatly assist the neonatologists in making appropriate decisions and improving the prognosis of neonates.

In recent years, studies on diagnostic biomarkers of NEC have attracted much attention. For example, the levels of serum amyloid A (SAA), IL-6, and IL-8 are elevated in NEC and are correlated with the severity of NEC; however they are non-specific biomarkers of NEC, and they may not appear early enough to be considered as predictive biomarkers [14–16]. The intestinal fatty acid binding protein (I-FABP) is thought to be a promising biomarker for NEC, but its use is limited due to medium sensitivity [14–16]. In addition, Aydemir et al. investigated the value of faecal calprotectin in diagnosis and predicting severity of NEC [17]. However, its usefulness as an early screening marker still remains unknown [14–17]. Faecal S100A12 begins to rise 10 days prior to the onset of disease and continues to increase until the day of onset [18]. However, its use as a predictive biomarker is limited due to high inter-individual and intra-individual variability in S100A12 faecal excretion [14,15,18]. And none of these biomarkers were evaluated to discriminate FPIES from NEC in neonates.

IL-27 is a heterodimeric cytokine comprised of the IL-27 p28 subunit and the Epstein-Barr virus-induced gene 3 (EBI3) protein, and it is primarily produced by antigen presenting cells [19]. IL-27 expression can be upregulated by toll-like receptor 4 (TLR4) signalling [20,21], which plays a critical role in the pathogenesis of NEC [2,22]. In addition, IL-27 also plays a central role in inflammatory intestinal diseases [19,23,24], therefore, IL-27 is supposed to be closely linked with NEC pathogenesis. Serum and intestinal tissue levels of IL-27 were significantly increased in experimental models of inflammatory bowel disease [19]. However, serum IL-27 levels were decreased in patients with allergic diseases, such as asthma and allergic rhinitis [25,26]. Therefore, we hypothesized that IL-27 could be a potential biomarker to differentiate NEC from FPIES. The primary objective of this study was to assess the usefulness of IL-27 for differentiating NEC from highly suspected early onset-FPIES (HSEO-FPIES) and to compare the diagnostic efficiency of IL-27 with the current laboratory indicators.

2. Materials and methods

2.1. Ethics approval

Human studies were reviewed and approved by the Clinical Research Ethics Committee of Chongqing Medical University (Registration number: 2019–12), and informed consent was obtained from all the guardians of participants according to the Declaration of Helsinki.

2.2. Study design and participants

This prospective study was carried out from June 2019 to December 2020, and all samples used in this study were obtained from the neonatal diagnosis centre of Children's Hospital. The research included a case-control study to explore whether the expression of IL-27 differed between neonates with NEC and those with HSEO-FPIES, and a validation cohort study to analyse the diagnostic efficiency of IL-27 for differentiating NEC from HSEO-FPIES. In the case-control study, neonates with NEC (n = 13), HSEO-FPIES (n = 9) and jaundice (control, n = 8) were enroled. In the validation cohort study, patients with bloody stools, vomiting, or abdominal distension who underwent radiologic or ultrasonographic examination were prospectively enroled. The exclusion criteria were as follows: (1) patients without any signs of pneumatosis intestinalis, portal venous gas, or pneumoperitoneum in any imaging examination (n = 17); (2) patients with congenital abdominal diseases, such as Hirschsprung's disease

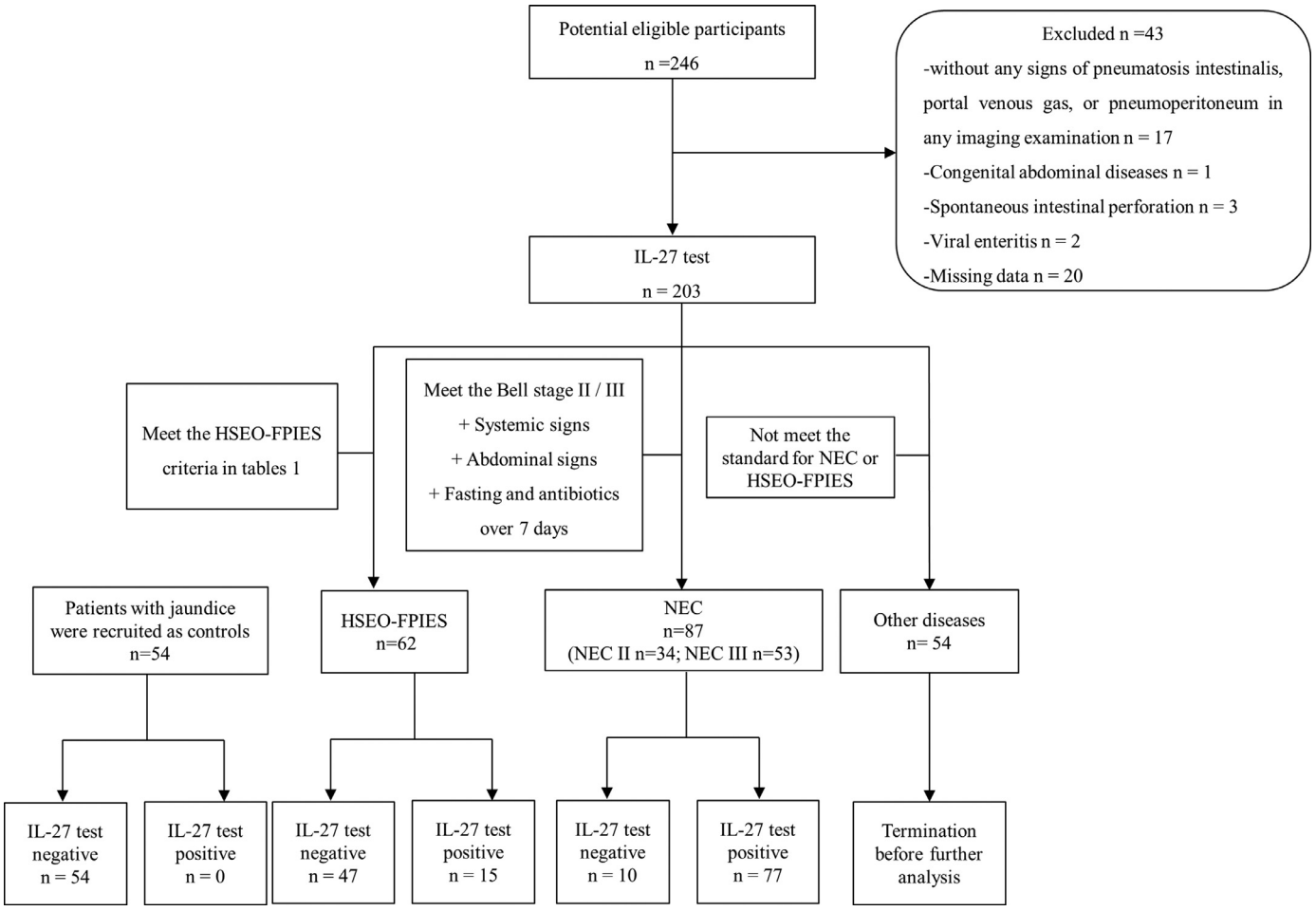

Fig. 1. Flow chart of sample collection and disease diagnosis. Abbreviations: HSEO-FPIES, highly suspected early onset food protein-induced enterocolitis syndrome; NEC, necrotising enterocolitis; IL, interleukin.

(n = 1); (3) surgical patients with spontaneous intestinal perforation (n = 3), (4) patients suspected of having viral enteritis, and enteric viruses were detected in the stool (n = 2); and (5) patients with missing data, which meant that the guardians of patients could not provide all the necessary information on diseases (n = 20). Initially, a total of 246 cases were included in the validation cohort study, but 43 neonates were excluded as they met the exclusion criteria. Then these patients were categorised into the NEC group (n = 87), HSEO-FPIES group (n = 62), and other diseases group (n = 54) according to the following diagnostic procedure. Patients with jaundice served as controls (n = 54) (Fig. 1).

2.3. Diagnosis and definition

The diagnostic process was in accordance with the following protocol: In the finally included 203 patients, we firstly judged whether the patients suffered from NEC according to the modified Bell's staging criteria II or III. Except for the intestinal signs with bloody stools, vomiting, or abdominal distension, patients in the NEC group should have received antibiotics and fasting for at least 7 days, show abdominal signs (such as hypoactive bowel sounds and abdominal tenderness) and have systemic signs (such as fever and apnoea) [1]. Then based on the adjusted International Consensus Guidelines, which recommend at least one major criterion plus at least three minor criteria (Table 1, [4,27,28]) it was judged whether the remaining patients met the criteria for HSEO-FPIES. Patients who did not meet the criteria for NEC or HSEO-FPIES were categorised as having other diseases (n = 54), which were not analysed. In addition, due to the ethical concerns, we were unable to collect venous blood from healthy newborns for research. Therefore, we selected neonates with only mild jaundice as controls (n = 54) to determine the basic expression of IL-27. Eventually, diagnoses of NEC and HSEO-FPIES were confirmed by

Table 1
Diagnostic criteria for highly suspected early onset food protein-induced enterocolitis syndrome (HSEO-FPIES).

Criteria	Defining features
Major criteria	(1) Vomiting or bloody stools after ingestion of the suspected food and absence of fever (> 38 °C) with any cutaneous reaction (urticaria, hives, and/or angioedema)
	(2) Modification of the infants' diet (for formula feeding) or mother's diet (for breast feeding) resulting in symptom resolution
	(3) Antibiotics were used for less than three days and fasting was ceased within three days
Minor criteria	(1) A second (or more) episode of repetitive symptoms after eating a similar formula or mother eating suspected trigger food (for breast feeding)
	(2) Repetitive symptoms after eating a different type of formula
	(3) Extreme lethargy with any suspected reaction
	(4) Marked pallor with any suspected reaction
	(5) Need for emergency department visit with any suspected reaction
	(6) Need for intravenous fluid support with any suspected reaction
	(7) Diarrhoea within 24 h
	(8) Hypotension
	(9) Hypothermia

two senior neonatologists who had been working for more than 15 years in the clinic and were not aware of the result of IL-27.

2.4. Sample collection and detection

In the case-control study, blood samples were collected when the diagnoses of NEC or HSEO-FPIES were established. In the validation cohort study, blood samples were obtained when the neonates met the inclusion criteria. Next, continuous monitoring was performed for the collection of blood samples until the patient was discharged. Peripheral blood was collected by venipuncture, and serum was isolated by centrifugation at 3000 rpm for 3 min at 4 °C and was stored in 200 μL aliquots at −80 °C for IL-27 and IL-6 testing. Blood samples were collected in K2-ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes to perform the complete blood count (CBC), WBC differential count, and CRP tests.

IL-27 and IL-6 levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (DY2526−05, R&D Systems, Minneapolis, MN, USA and 430, 504, BioLegend, San Diego, CA, USA). Assays were performed according to the manufacturers' instructions. CBC and WBC differential counts were determined using an Automated hematology analyser (SYSMEX-500i, Japan). CRP levels were determined with the QuikRead Go CRP system (Orion Diagnostica, Finland), and a sensitivity of 8 mg/L was set as the detection limit.

2.5. Sample size

According to the serum IL-27 levels of NEC group and HSEO-FPIES group in the case-control study, the sample size was estimated by the software PASS 11.0 (Hintze, J. (2011). PASS 11. NCSS, LLC. Kaysville, Utah, USA. www.ncss.com.), α = 0·05, and 1-β = 0·90. The estimated sample size was 50 cases per group, and the sample size in this study was larger than the estimated required number of cases.

2.6. Statistical analysis

Normal analysis was performed by the Kolmogorov-Smirnov test on quantitative data, including the demographic data, clinical characteristics, and biomarker concentrations. Most of the data showed a skewed distribution. Therefore, we used medians and interquartile ranges (IQR) to describe these data. The Kruskal-Wallis test was used to compare the demographic data, clinical characteristics, and biomarker levels amongst the three or three above study groups, and the significant differences were found, we then performed the Mann-Whitney U test between the two groups. Qualitative or categorical variables were expressed as frequencies and proportions. Proportions were compared using the $\chi 2$ test. Spearman's correlation coefficient was used to evaluate the correlation between IL-27 and other laboratory indicators. All statistical tests were two sided and were performed at a significance level of $p > 0{\cdot}05$ using the SPSS version 20.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, USA).

To compare the diagnostic value of biomarkers, receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was determined. Optimal cut-off points were determined based on ROC curves. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and positive or negative likelihood ratios (±LR) were calculated for the parameters used in the differential diagnosis. These analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA), Medcalc version 15.2.2 (MedCalc Software, Ostend, Belgium), and SAS9.0 (SAS Institute, Cary, NC, USA).

2.7. Role of funding source

The funding source had no role in writing, data collection, analysis, or interpretation, or any aspect pertinent to the study. The corresponding authors had full access to all data and had final responsibility for the decision to submit the manuscript for publication.

3. Results

3.1. Case-control study to determine if serum levels of IL-27 can potentially discriminate NEC from HSEO-FPIES

3.1.1. Patient characteristics

Clinical characteristics of neonates in the case-control study are summarised in Supplementary Table 1. Neonates in the NEC group had a lower gestational age (p = 0·036), lower birth weight (p = 0·051), and longer hospital stay (p = 0·00020) compared with those in the HSEO-FPIES group. However, there were no significant differences in Apgar scores at 1 and 5 min, sex distribution, delivery mode, or oxygen requirements between the two groups.

3.1.2. Serum levels of IL-27

IL-27 levels were significantly increased in the NEC group compared to the HSEO-FPIES group (p = 0·00050), and they were higher in the HSEO-FPIES group than in the control group (p = 0·00010) (Fig. 2). These results indicated the potential application of IL-27 as a biomarker for differentiating NEC from HSEO-FPIES.

3.2. A prospective cohort study to validate the diagnostic utility of IL-27

3.2.1. Patient characteristics

Clinical characteristics of neonates in the validation cohort study are shown in Table 2. Compared with neonates in the HSEO-FPIES group, neonates in the NEC group had a lower gestational age ($p < 0{\cdot}0001$), lower birth weight ($p < 0{\cdot}0001$), lower Apgar score at 1 min (p = 0·028), higher proportions of anaemia ($p < 0{\cdot}0001$) and RBC transfusions (p = 0·019), and longer hospital stay ($p < 0{\cdot}0001$). These trends reached statistical significance, but they were frequently observed in NEC cases [2]. There were no significant differences in the Apgar score at 5 min (p = 0·41), sex distribution (p = 0·90), the feeding patterns (p = 0·083), delivery mode (p = 0·12), and the proportion of prolonged premature rupture of membrane over 18 h (P-PROM, p = 0·86) and oxygen requirement (p = 0·18) between the NEC and HSEO-FPIES groups.

3.2.2. Serum levels of IL-27

Serum IL-27 levels were assessed to determine any potential correlations with postnatal and gestational age or birth weight of controls. IL-27 levels were found to be similar between males and females (p = 0·97) as well as between different postnatal ages (p = 0·65) in controls(Fig. 3a,b). There was no significant correlation of IL-27 levels with gestational age (p = 0·32) or birth weight (p = 0·93) in the control group (Fig. 3c,d). IL-27 levels remained high from 0 to 3 days of NEC onset, and then they declined slightly but still remained high in the NEC group (Fig. 3e). In contrast, there were no significant trends in IL-27 levels in the HSEO-FPIES group (Fig. 3f). IL-27 levels were significantly higher in the NEC group than in the HSEO-FPIES group ($p < 0{\cdot}0001$), and they were higher in the HSEO-FPIES group than in the control group ($p < 0{\cdot}0001$) (Fig. 3g). These results indicated that IL-27 was a potential marker to differentiate NEC from HSEO-FPIES.

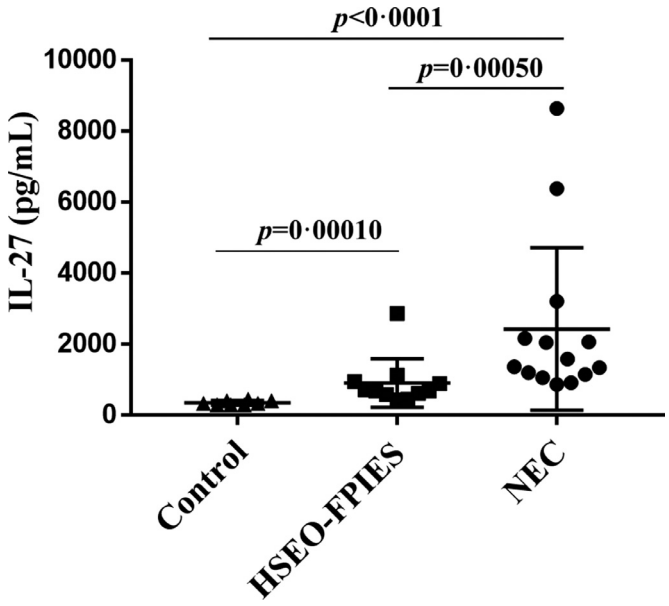

Fig. 2. Serum interleukin (IL)−27 levels in the case-control study. Abbreviations: HSEO-FPIES, highly suspected early onset food protein-induced enterocolitis syndrome; NEC, necrotising enterocolitis. The Kruskal-Wallis test was used to compare the IL-27 levels amongst the three groups, then the Mann-Whitney U test was performed between the two groups.

3.2.3. Levels of current laboratory biomarkers and their correlation with IL-27

Next, the levels of IL-6, CRP, WBC, N, L, neutrophil to lymphocyte ratio (N/L), and PLT were determined on the day of hospital admission. X-ray scores were also analysed. Compared with neonates in the HSEO-FPIES group, WBC (p = 0·016), L (p < 0·0001), and PLT (p < 0·0001) levels were significantly decreased and all other markers were significantly elevated in neonates in the NEC group (Supplementary Fig. 1a). Additionally, IL-27 levels weakly correlated with IL-6 (r = 0·33, p = 0·0031), CRP (r = 0·35, p < 0·0001), WBC (r=−0·20, p = 0·018), PLT (r=−0·23, p = 0·0073), and X-ray scores (r = 0·29, p = 0·0013), but they did not correlate with the N (r = 0·023, p = 0·80), L (r=−0·039, p = 0·67) and N/L ratio (r = 0·15, p = 0·11) (Supplementary Fig. 1b).

3.2.4. Diagnostic utility of IL-27 to discriminate NEC from HSEO-FPIES

The diagnostic efficiency of IL-27 to discriminate NEC from HSEO-FPIES was assessed, and the AUC for IL-27 was found to be the largest (0·878) amongst the given biomarkers. The cut-off value, sensitivity, and specificity were 1055·17 pg/mL, 88·37%, and 75·81%, respectively. Although IL-6 and CRP were more specific indicators (98·04%, 94·64%, respectively), they showed much lower sensitivities (44·64%, 64·10%, respectively) than IL-27 (Table 3). Taken together, IL-27 provided the greatest diagnostic utility to discriminate NEC from HSEO-FPIES amongst all given indicators (Fig. 4). This strongly suggests that IL-27 is a potential biomarker for differentiating NEC from HSEO-FPIES.

3.2.5. Diagnostic value of IL-27 in combination with the current laboratory biomarkers to differentiate NEC from HSEO-FPIES

The performance of different combinations of biomarkers was evaluated to determine whether their ability to distinguish NEC from HSEO-FPIES could be improved. The combination of IL-27 with IL-6, CRP, or N/L generated AUC values of 0·898, 0·903, and 0·893, respectively, and these AUC values were larger than that for IL-27 alone (AUC = 0·878). The optimal result was obtained using the combination of IL-27 and CRP (AUC = 0·903) (Table 4).

3.3. The link between IL-27 and NEC severity

Increased levels of circulating IL-27 were associated with the severity of NEC. When compared with non-surgically treated neonates, significantly higher IL-27 levels were observed in neonates who received surgical treatment in the NEC group (p = 0·00070) (Fig. 5a). In addition, IL-27 concentrations were significantly increased in neonates in the NEC group who were diagnosed with peritonitis (p = 0·025), sepsis (p < 0·0001), or shock (p = 0·00080) compared with those who did not experience these episodes (Fig. 5b−d). These results demonstrated that highly elevated IL-27 levels were linked with a poor prognosis of NEC.

Table 2
Clinical characteristics of neonates in the validation cohort study.

Demographic/Clinical Characteristics	NEC (n = 87)	HSEO-FPIES (n = 62)	Control (n = 54)	p-values		
				NEC VS HSEO-FPIES	NEC VS Control	HSEO-FPIES VS Control
Gestational age, w	35·00 (33·00−38·00)	38·00 (38·00−40·00)	39·00 (37·00−39·00)	< 0·0001	< 0·0001	0·13
Birth weight, g	2300 (1730−3000)	3200 (2980−3500)	3250 (2890−3440)	< 0·0001	< 0·0001	0·55
Sex, female, n (%)	43 (49·43)	30 (48·39)	26 (48·15)	0·90	0·88	0·98
Onset of illness, d	6·00 (3·00−10·00)	9·00 (4·50−12·50)	2·00 (0·75−3·00) #	0·037	< 0·0001	< 0·0001
Nutrition management				0·083	0·0080	0·11
-Exclusive breastfeeding, n (%)	22 (25·29)	15 (24·20)	23 (42·60)	–	–	–
-Exclusive formula feeding, n (%)	33 (37·92)	14 (22·58)	8 (14·80)	–	–	–
-Mixed feeding, n (%)	32 (36·79)	33 (53·22)	23 (42·60)	–	–	–
Caesarean section, n (%)	69 (79·31)	43 (69·35)	14 (25·93)	0·12	< 0·0001	< 0·0001
P-PROM, n (%)	5 (5·75)	4 (6·45)	2 (3·70)	0·86	0·59	0·51
Apgar score, 1 min	9·00 (8·00−10·00)	10·00 (9·00−10·00)	10·00 (9·00−10·00)	0·028	0·014	0·41
Apgar score, 5 min	10·00 (9·00−10·00)	10·00 (10·00−10·00)	10·00 (10·00−10·00)	0·41	< 0·0001	< 0·0001
Oxygen requirement, n (%)	39 (44·83)	21 (33·87)	5 (9·26)	0·18	< 0·0001	0·0020
Anaemia, n (%)	57 (65·52)	19 (30·65)	2 (3·70)	< 0·0001	< 0·0001	< 0·0001
RBC transfusion*, n (%)	13 (14·94)	2 (3·23)	0 (0·00)	0·019	< 0·0030	0·18
Hospitalization length, d	21·00 (10·75−31·00)	9·00 (6·25−11·00)	5·00 (3·00−7·00)	< 0·0001	< 0·0001	< 0·0001

Data were described using medians and interquartile ranges (IQR). Qualitative or categorical variables were expressed as frequencies and proportions. Abbreviations: HSEO-FPIES, highly suspected early onset food protein-induced enterocolitis syndrome; NEC, necrotizing enterocolitis; P-PROM, prolonged premature rupture of membrane (PROM > 18 h); RBC, red blood cell.

#, onset of illness for control group indicated admission age; *, RBC transfusion before the confirmed diagnosis of NEC. The Kruskal-Wallis test was used to compare the demographic data, clinical characteristic among the three study groups, then the Mann-Whitney U test were performed between the two groups. Proportions were compared using the $\chi 2$ test.

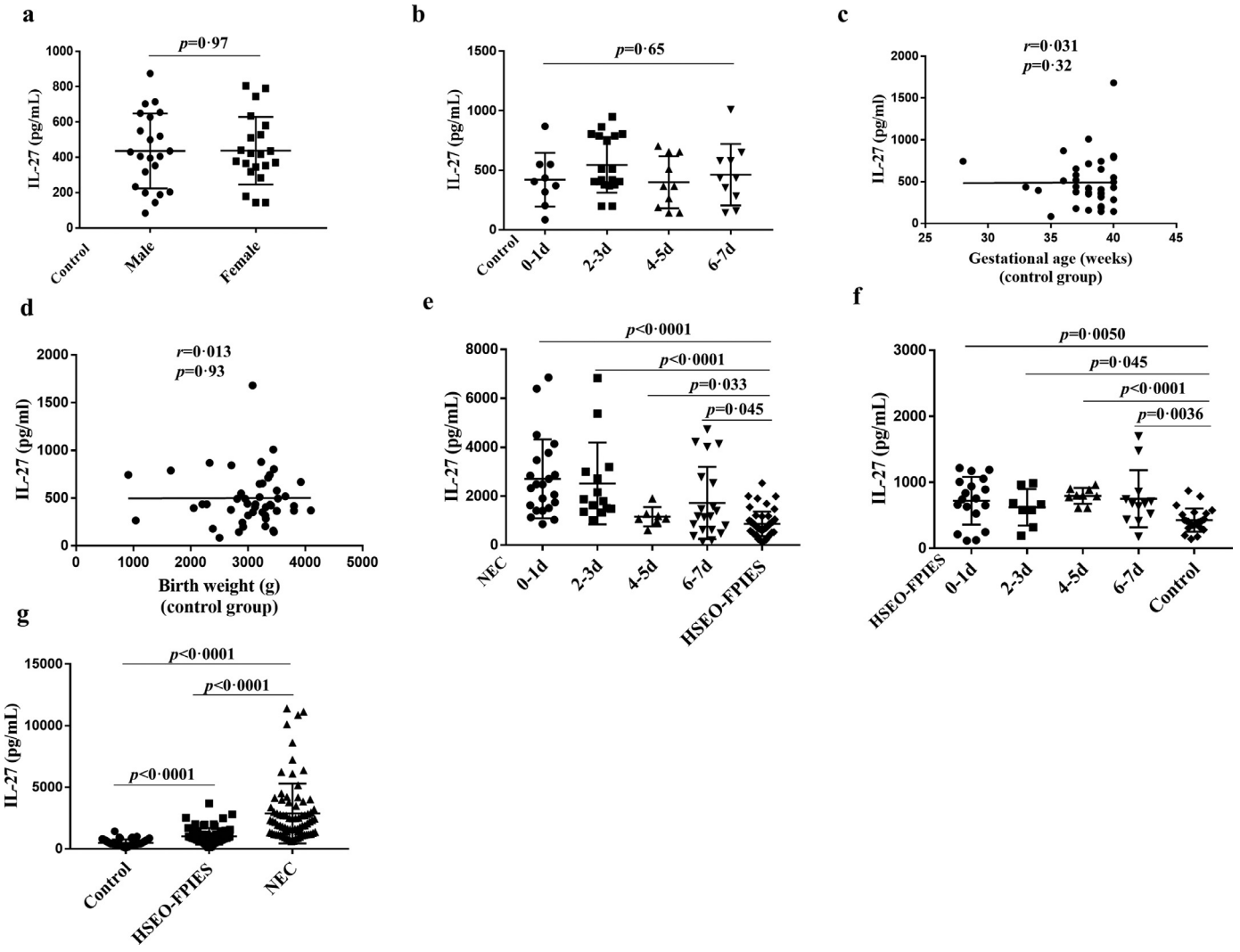

Fig. 3. Serum levels of IL-27 in the validation cohort study. (a) IL-27 classified by gender in the control group. (b) IL-27 levels for the first seven days after birth in the control group. Correlation of IL-27 with (c) gestational age and (d) birth weight in control patients. (e) IL-27 levels from day 0 of NEC onset to day 7. (f) IL-27 levels from day 0 of HSEO-FPIES onset to day 7. (g) IL-27 levels amongst the three groups of the validation cohort study. Abbreviations: IL, interleukin; HSEO-FPIES, highly suspected early onset food protein-induced enterocolitis syndrome; NEC, necrotising enterocolitis. Three or above three groups comparisons were performed by the Kruskal-Wallis test, then two groups comparisons were performed by the Mann-Whitney U test,and the correlations of IL-27 levels with postnatal and gestational age or birth weight were performed by spearman's correlation coefficient.

Table 3
AUC and optimal cut-off points with their corresponding validity indexes and predictive values in the cohort study.

Indicators	AUC (95% CI)	Se (%)(95% CI)	Sp (%) (95% CI)	J (%)	PPV (%)	NPV (%)	+LR	-LR	Cut-off value
IL-27	0·878 (70·10–91·30)	88·37 (79·7–94·3)	75·81 (63·3–85·8)	0·64	83·5	82·5	3·65	0·15	1055·17
IL-6	0·761 (0·40–0·70)	44·64 (31·3–58·5)	98·04 (89·6–100·0)	0·43	96·20	61·70	22·77	0·56	201·70
CRP	0·800 (53·50–73·40)	64·10 (49·8–73·7)	94·64 (85·9–98·9)	0·59	94·30	65·40	11·97	0·38	8·00
WBC	0·637 (44·80–64·10)	39·02 (28·4–50·4)	98·36 (91·2–100·0)	0·37	97·00	54·50	23·80	0·62	6·14
N	0·765 (54·80–77·10)	57·63 (44·1–70·4)	86·21 (74·6–93·9)	0·44	81·00	66·70	4·18	0·49	0·53
L	0·782 (56·60–80·10)	66·10 (52·6–77·9)	80·36 (67·6–89·8)	0·46	78·00	69·20	3·37	0·42	0·38
N/L	0·781 (58·70–81·70)	66·67 (52·9–78·6)	82·64 (70·1–91·3)	0·49	79·20	71·20	3·80	0·40	1·36
PLT	0·729 (49·50–74·30)	70·73 (59·6–80·3)	64·52 (51·3–76·3)	0·35	72·50	62·50	1·99	0·45	363·00

Abbreviations: AUC, area under the ROC curve; Cut-off, optimal cut-off points; +LR, positive likelihood ratio; -LR, negative likelihood ratio; Se, sensitivity; Sp, specificity; J, Youden index; PPV, positive predictive value; NPV, negative predictive value; IL, interleukin; CRP, C-reactive protein; WBC, white blood cell; N, neutrophils; L, lymphocytes; N/L, neutrophil to lymphocyte ratio; PLT, platelet count.

4. Discussion

The initial clinical manifestations and abdominal imaging findings of neonates with NEC and FPIES are sometimes similar. It is sometimes clinically difficult to differentiate between these two disease entities. In this study, we found that serum IL-27 levels were significantly higher in both NEC and FPIES groups compared to the control group, and IL-27 level in the NEC group was significantly higher than that in the FPIES group. IL-27 was a potential biomarker to differentiate NEC from HSEO-FPIES with the largest AUC of 0·878 amongst the tested indicators, sensitivity of 88·37%, and specificity of 75·81%.

Our results also showed that Il-27 combined with IL-6, CRP, or N/L yielded larger AUC values than IL-27 alone. The optimal AUC was obtained by the combination of IL-27 with CRP. This can be explained

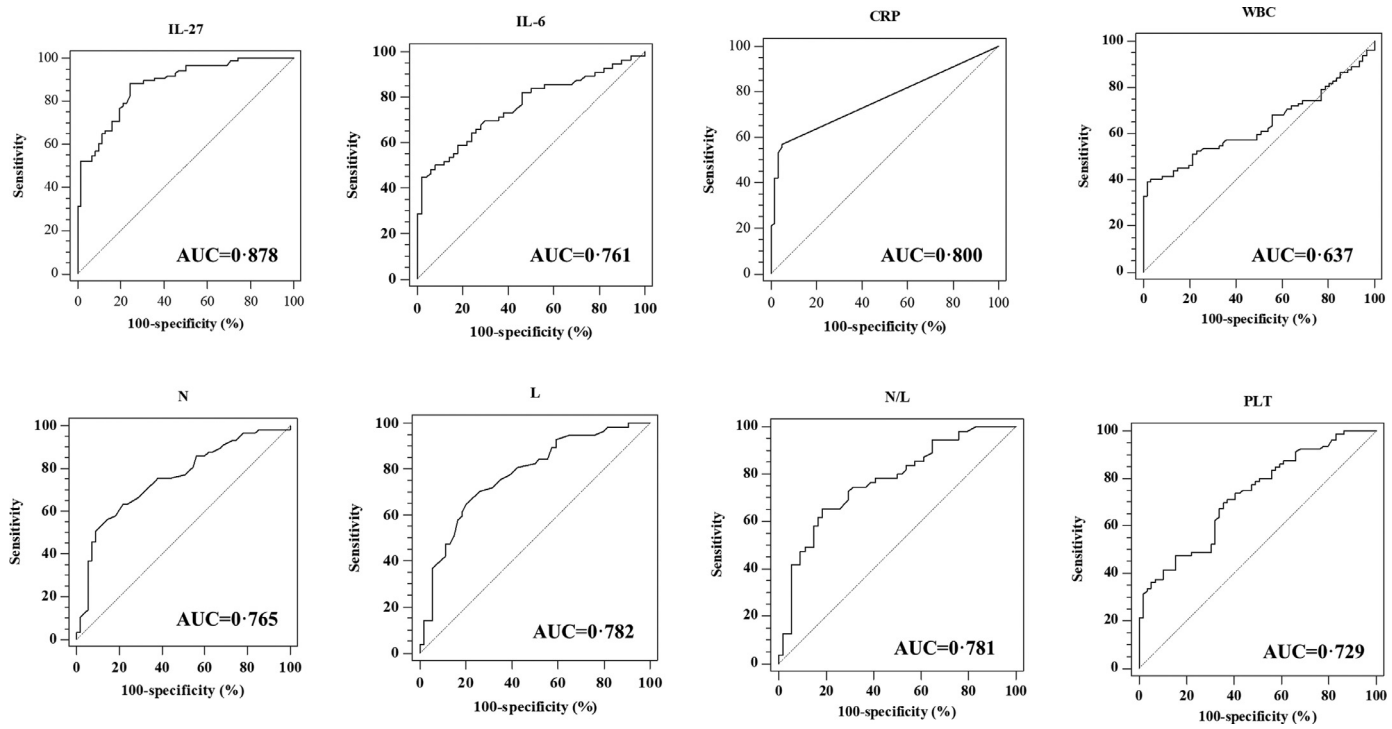

Fig. 4. ROC curves for biomarkers to differentiate NEC from HSEO-FPIES. Abbreviations: ROC, receiver operating characteristic; HSEO-FPIES, highly suspected early onset food protein-induced enterocolitis syndrome; NEC, necrotising enterocolitis; IL, interleukin; CRP, C-reactive protein; WBC, white blood cell count; N, neutrophils; L, lymphocytes; N/L, neutrophil to lymphocyte ratio; PLT, platelets.

Table 4
Diagnostic efficiency of IL-27 in combination with the current laboratory markers for differentiating NEC from HSEO-FPIES.

Indicators	Cut-off	Se (95% CI)	Sp (95% CI)	+LR	-LR	AUC (95% CI)	*p*-values
IL-27 + IL-6	≥ 1 positive	94·32 (87·2–98·1)	72·31 (59·8–82·7)	3·41	0·079	0·898 (0·839- 0·941)	< 0·0001
	= 2 positive	52·27 (41·4–63·0)	98·46 (91·7–100·0)	33·98	0·480		
IL-27+ CRP	≥ 1 positive	94·32 (87·2–98·1)	69·23 (56·6–80·1)	3·07	0·082	0·903 (0·845 –0·945)	< 0·0001
	= 2 positive	60·23 (49·2–70·5)	98·46 (91·7–100·0)	39·15	0·400		
IL-27 + WBC	≥ 1 positive	88·64 (80·1–94·4)	70·77 (58·2–81·4)	3·03	0·160	0·837 (0·768–0·891)	< 0·0001
	= 2 positive	31·82 (22·3–42·6)	98·46 (91·7–100·0)	20·68	0·690		
IL-27 + *N*/L	≥ 1 positive	96·59 (90·4–99·3)	61·54 (48·6–73·3)	2·51	0·055	0·893 (0·833–0·937)	< 0·0001
	= 2 positive	64·77 (53·9–74·7)	95·38 (87·1–99·0)	14·03	0·370		
IL-27 + PLT	≥ 1 positive	94·32 (87·2–98·1)	44·62 (32·3–57·5)	1·70	0·130	0·838 (0·770–0·892)	< 0·0001
	= 2 positive	64·77 (53·9–74·7)	92·31 (83·0–97·5)	8·42	0·380		

Abbreviations: HSEO-FPIES, highly suspected early onset food protein-induced enterocolitis syndrome; NEC, necrotizing enterocolitis; AUC, area under the ROC curve; Cut-off, optimal cut-off points; +LR, positive likelihood ratio; -LR, negative likelihood ratio; Se, sensitivity; Sp, specificity; IL, interleukin; CRP, C-reactive protein; WBC, white blood cell count; N/L, neutrophil to lymphocyte ratio; PLT, platelet count.

by the following reasons: Owing to its long doubling time, the CRP level hardly increased in the early stage of inflammation, but it was constantly elevated if the inflammation persisted. On the contrary, we found the IL-27 level increased on day 1−3 of NEC. The combination of early marker IL-27 and late inflammatory marker CRP increased the diagnostic value.

Interestingly, it was also found that IL-27 levels were linked to NEC severity. In the current study, IL-27 levels were significantly and positively related to X-ray scores, and IL-6 and CRP levels, but they were negatively related to PLT and WBC levels. These results were consistent with previous reports that X-ray scores, and IL-6 and CRP levels were positively correlated with NEC severity, but low PLT and WBC levels indicated more serious cases of NEC [5]. IL-27 levels were markedly elevated in stage II of NEC, and the median values reached 1580·73 pg/mL and 2491·00 pg/mL in stages II and III, respectively. In the NEC group, significantly higher IL-27 levels were observed in surgery-related neonates compared with nonsurgical neonates. IL-27 levels were significantly increased in neonates who had NEC with peritonitis, sepsis, or shock compared to neonates with NEC who did not experience these episodes. These results were also consistent with previous reports suggesting that IL-27 is a potential biomarker for the diagnosis of neonatal sepsis and bacterial infection [29,30]. Therefore, high levels of IL-27 may suggest a poor prognosis and serious complications.

In the validation cohort study, there were statistically significant differences between the NEC and HSEO-FPIES groups in terms of gestational age and birth weight, but IL-27 levels were not significantly correlated with these parameters. Also, circulating IL-27 levels were independent of postnatal age and sex. However, a previous study indicated that EBI3 and IL-27p28 expression levels were significantly increased from day 4 post-birth in mice [31]. This may indicate that IL-27 is expressed differently in humans and mice, and the sample size will be increased to examine this difference in expression in our future research.

IL-27 plays an important role in many diseases. Evidence suggests that IL-27 exhibits both pro- and anti-inflammatory effects in

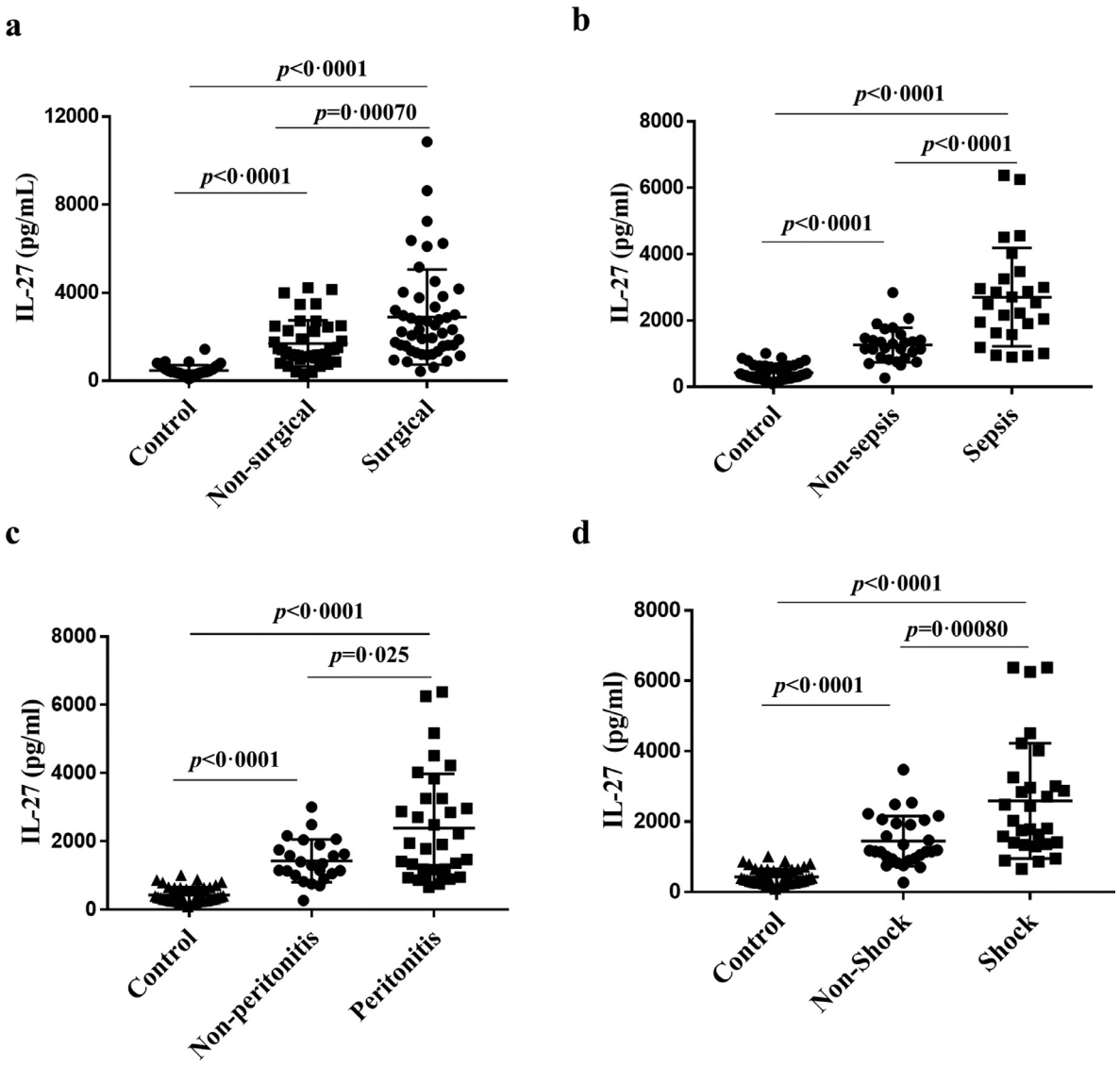

Fig. 5. The link between IL-27 and NEC severity. (a) IL-27 levels in surgical NEC neonates (n = 49), non-surgical NEC neonates (n = 38), and controls (n = 54). (b, c, d) IL-27 levels in patients in the NEC group who were diagnosed with peritonitis (n = 34), sepsis (n = 26), or shock (n = 29), those who did not experience these episodes, and controls (n = 54). Abbreviations: IL, interleukin; NEC, necrotising enterocolitis. The three groups' comparisons were performed by the Kruskal-Wallis test, then two groups' comparisons were performed by the Mann-Whitney U test.

different effective cells [23]. Some studies have reported that IL-27 levels were decreased in allergic diseases [25,26,32]. Allergic rhinitis, asthma, and FPIES are T cell-related hypersensitivity disorders [33–35]. However, unlike allergic rhinitis and asthma, which are characterized by elevated IgE, many patients with FPIES may have low levels of IgE [35]. This indicated that the pathogenesis of FPIES may be in some aspects different from that of allergic rhinitis and asthma. The expression of IL-27 in FPIES has not been reported. Unlike in the above allergic diseases, in our research, IL-27 expression was higher in the FPIES group compared to the control group; however, the mechanism may require further study.

There are some limitations to our study. First, all cases were recruited from only one hospital, the Children's Hospital of Chongqing Medical University, and results from a multicentre study would carry more weightage. Second, the control group included neonates with mild jaundice rather than healthy newborns due to ethical concerns. Third, the FPIES group consisted of neonates with HSEO-FPIES because oral food challenges, the gold standard for diagnosis, cannot be performed in neonates. Therefore, we used clinical criteria and labelled the group as "highly suspected early-onset food protein-induced enterocolitis syndrome"; however, further research is needed prior to the clinical application of our results. Fourth, the GA < 33 weeks and very low-birth-weight (VLBW) in NEC cases was lower than that in some studies, and several factors can contribute to this situation. In China, the gestational age of neonates with NEC is greater than that in most countries, which is in accordance with the current situation of low breastfeeding rates and underdeveloped technology of preterm delivery support. Our centre did not have a maternal ward; all VLBW neonates were transferred from other hospitals, including some level I or II hospitals. To our knowledge, the early resuscitation strategy is significant for VLBW babies, and as a developing country, some of the level I or II hospitals did not have adequate facilities (both equipment and skilled doctors) to deal with VLBW neonates in the early period, which could result in a relatively high mortality of VLBW neonates. As a consequence, the morbidity of NEC would decrease in VLBW neonates since the NEC tends to occur several weeks after birth in VLBW neonates. In addition, according to data from the China Neonatal Network (CHNN), about 12% of neonates with GA < 31 weeks are discharged against medical advice due to financial difficulty while 23% of extremely low-birth-weight

(ELBW) neonates do not receive complete care in our hospital due to financial difficulty [36]. In fact, according to the data from CHNN, from 2015 to 2018, in the 25 tertiary neonatal centres included, only 87 cases of confirmed NEC were identified with GA < 32 weeks [36]. Finally, the exclusive breastfeeding rate in China is much lower than that in developed countries because the human milk bank is rarely found in China and limited donated human milk would be mainly provided to VLBW neonates. Breastfeeding is a protective factor against NEC; thus, a low breastfeeding rate in moderate or late preterm infants may contribute to a relative higher prevalence of NEC in this population. The above reasons may help explain why our NEC cases had a relatively higher GA. In spite of the above facts, there was no significant difference in the expression of IL-27 at GA > 33 weeks and GA ≤ 33 weeks (p = 0·88) in the NEC group. Therefore, our findings might potentially differentiate between classical NEC and FPIES in VLBW neonates.

5. Conclusions

To our knowledge, this is the first study to explore the value of a biomarker to discern NEC from HSEO-FPIES in a relatively large population of neonates. IL-27 is sensitive for discerning NEC from HSEO-FPIES, and it has good diagnostic efficacy. This study provides a new perspective for the early differential diagnosis between NEC and HSEO-FPIES.

Contributors

Yu He: study design, data analysis, funding acquisition, validation, data interpretation; Wenchun Xu: study design, writing, validation, visualisation; Yuhong Qi: literature search, figures, study design, data collection, data analysis, data interpretation, writing, validation; Chan Liu , Xin Zhong, Xueling Ma, Jie Zhou: sample collection; Yuan Shi, Yibing Yin, Xuemei Zhang: study design.

Declaration of Competing Interest

All authors declare that a patent related to “Cytokine IL-27 as a differential diagnostic marker for NEX and FPIES and its application” has been filed.

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Data sharing statement

Data generated or used in this study are available from the corresponding authors upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103607.

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