Clinical Value of Serum Interleukin-33 Biomarker in Infants With Neonatal Cholestasis

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See ''Just Another Needle in a Haystack? Can a Single Cytokine Improve Medical Care in Biliary Atresia?'' by Junge on page 278.

ABSTRACT

Objectives: The present study aimed to estimate the value of serum interleukin-33 (IL-33) levels in infants with cholestasis, correlate serum IL-33 levels with the clinicopathological profile of infants with cholestasis, and compare its level with that of healthy infants who served as control.

Methods: Sixty infants with cholestasis were enrolled in the present study and divided into biliary atresia (BA) group and non-BA group, in addition to 30 healthy infants as a control group. All infants were analyzed for their clinical and biochemical features, histopathological profile, and serum level of IL-33 by enzyme-linked immune sorbent assay.

Results: Serum level of IL-33 in BA group (median 48.0, interquartile range: 28.9–106.2) was significantly higher than that of the non-BA group (median 17.3, interquartile range: 13.7–18.8 pg/mL) and both were higher than that of the control group. There was a positive correlation between serum IL-33 and aspartate aminotransferase, alanine aminotransferase, bilirubin (total and direct) levels, and fibrosis stage among the BA group. Serum IL-33 at a cut-off value of 20.8 pg/mL can detect BA with a specificity of 95% and a sensitivity of 96.7%.

Conclusion: The significantly higher production of IL-33 in patients with BA compared to non-BA suggests a potential role of IL-33 for initiation and progression of the disease process, also, IL-33 may have a diagnostic role in infants with BA.

Key Words: biliary atresia, cholestasis, interleukin-33

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What Is Known

- Interleukin-33 is an important factor involved in the pathogenesis of liver damage during acute and chronic hepatitis.
- Interleukin-33 attracts type 2 innate lymphoid cells, which activate the profibrogenic function of hepatic stellate cells.

What Is New

- The higher production of interleukin-33 in patients with biliary atresia compared to nonbiliary atresia suggests a potential role of interleukin-33 for initiation and progression of the disease process.
- Interleukin-33 may have a diagnostic value in infants with biliary atresia.

The term ''neonatal cholestasis'' is frequently referred to as a cholestatic liver disease that occurs at birth and develops within the first few months of life. In clinical practice, these illnesses usually become apparent within the initial 2 months of life, though, similar diagnostic thoughts apply for infants whose cholestasis is recognized after 2 months of age (1).

Biliary atresia (BA) has an occurrence of only 1 in 10 to 19,000 in Europe and North America, other diseases (eg, the genetic conditions, Alagille syndrome, and progressive familial intrahepatic cholestasis) are less common (2).

Hepatocellular injury is the main mediator of hepatic fibrosis as it primes to inflammation, with the enrolment and activation of innate lymphocytes, neutrophils, hepatic stellate cells (HSCs), and T lymphocytes (3). Activated HSCs yield inflammatory chemokines, express cell adhesion molecules, activate lymphocytes, and release extracellular matrix, contributing to hepatic fibrosis (4). Prominently activation of a Th2 response is known to origins fibrosis of the liver and additional organs (5).

Interleukin-33 (IL-33), an IL-1-related cytokine, has appeared as an important cytokine for bringing Th2 cytokine production. IL-33 is released on cell death, and augmented IL-33 can be noticed in severe inflammation (3,6). IL-33 required the IL-33/suppressor of tumorigenicity 2 (ST2) receptor to yield proinflammatory and Th2 cytokines (7). To date, it is supposed that IL-33 assists as an ''alarmin'' released by stressed hepatocytes. IL-33 then attracts type 2 innate lymphoid cells (ILC2), which activate the profibrogenic activation of HSCs through mediators such as IL-13 (8). So the aim of the present study was to evaluate the value of serum IL-33 levels in infants with cholestasis, correlate serum IL-33 levels with the clinicopathological profile of infants with

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cholestasis, and compare its level with that of healthy infants who served as control.

SUBJECTS AND METHODS

Subjects

This cross-sectional case-controlled study was performed on 60 infants with cholestasis attending the Pediatric Hepatology Clinic of Benha University Hospitals and National Liver Institute, Menoufia University from August 2017 to January 2019. After informed consent was obtained from all parents, they were divided into 2 groups.

Biliary atresia group included 30 infants diagnosed as BA that were based on laparoscopy with surgical cholangiography (absence of dye excretion on intraoperative cholangiogram after ruling out other causes of obstructive jaundice). Seventeen patients of them whose age ranged from 1 to 3 months underwent Kasai hepatoportoenterostomy.

The gall bladders or the remnants of gall bladders were found, but extrahepatic biliary duct could not be detected during the operation and liver biopsy and showed the typical histopathological features (eg, bile plugs, duct proliferation, giant cell transformation, periportal fibrosis, and canalicular and cellular bile stasis) (9).

Nonbiliary atresia included 30 infants with cholestasis without BA. Cholestasis was defined as conjugated bilirubin >20% of the total bilirubin or total bilirubin is more and 17 mg/dL (10).

The exclusion criteria included any patient with hepatotropic viruses, other chronic diseases that elevate serum IL-33 such as bronchopulmonary dysplasia, allergic airway diseases, inflammatory bowel diseases, and chronic inflammation.

Control group included 30 apparently healthy infants matching the patient groups for age and sex who were selected from the well-baby clinic. The present study was agreed by the Medical Ethical Committee of Benha University conferring to the World Medical Association Declaration of Helsinki (11).

Methods

All children were subjected to the following:

1. Full history taking and physical examination with stress on clinical presentations (jaundice, clay-colored stool, abdominal pain and distension, melena, and another bleeding such as hematuria, epistaxis, or bleeding gums) general examination, anthropometric measurements, and abdominal examination.
2. Abdominal ultrasonography for estimating the liver (span and texture), presence of ascites, and spleen span.
3. Laboratory investigations

Sampling: six milliliters of venous blood were obtained after biopsy by peripheral venipuncture under aseptic precautions from all participants and divided as follows: 1 mL of blood on 150 μL ethylene diamine tetracaetic acid to perform complete blood count by Sysmex-XP300. The rest of the blood was centrifuged for 10 minutes then the resultant serum was used for chemical testing: serum aminotransferases (aspartate aminotransferase [AST]-alanine aminotransferase [ALT]), gamma-glutamyl transferase, alkaline phosphatase, bilirubin, albumin, and total protein that were done by Biosystem A15 autoanalyzer (Barcelona, Spain).

TORCH antibodies for patients' groups only: toxoplasma immunoglobulin M and immunoglobulin G antibodies were tested by enzyme-linked immune sorbent assay (ELISA) kit (Pishtaz Teb Zaman Diagnostics, Iran). Herpes simplex virus 1 and 2, cytomegalovirus (CMV), and rubella antibodies were tested using ELISA kits (Diapro Diagnostic Bioprobes, Italy). CMV antibody-positive infants were tested by CMV-DNA polymerase chain reaction.

The rest of the serum was stored at −20°C for measurements of serum IL-33 level by ELISA sandwich technique purchased from the Sunredbio Co (Shanghai, China) with (Cat No: 201-12-0045).

(1) Liver biopsy: Ultrasound-guided liver biopsy was done for all patients using the Menghini aspiration needle (Hepafix Luer Lock Melsungen AG, 3409 Mel-Sungen, and Germany). The biopsy specimen was fixed in formalin and embedded in paraffin. Five-micrometer thick sections were cut, mounted on a glass slide, and stained with hematoxylin and eosin to evaluate the histological activity of hepatitis using Ishak et al (12). Hepatic activity index (HAI) index, also stained with Masson trichrome to assess the fibrosis stage. The histopathologist who reported the liver histology was blinded to the IL-33 values.
(2) Assess disease severity by Child-Pugh score (13) and pediatric end-stage liver disease score (14) for participants younger than 12 years of age.

Statistical Analysis

The collected data were analyzed using SPSS version 16 software (SPSS Inc, Chicago, IL). Chi-square test (χ^2) or Fisher exact test were used to analyze categorical variables. Quantitative data were tested for normality by the Shapiro-Wilk test, assuming normality at $P > 0.05$. Nonparametric variables among 2 independent groups were analyzed using Mann-Whitney U test. The difference among 3 independent means was analyzed by analysis of variance for parametric variables or Kruskal-Wallis test for nonparametric ones. Spearman correlation coefficient (rho) was used to assess the linear association between nonparametric variables. Receiver operating characteristic curve was used to determine the cutoff value of IL-33 with optimum sensitivity and specificity in the detection of BA. ($P < 0.05$ was considered significant). Multiple regression analysis was run to detect the predictors. Binary logistic regression was used for IL-33 as a predictor of BA among patients with neonatal cholestasis.

RESULTS

The present study included 90 infants who were divided into 3 groups; BA: included 30 infants, they were (16 girls/14 boys) with mean age ± standard deviation (SD) 4.6 ± 2.8 months. Non-BA cholestasis group included 30 infants, they were (15 girls/15 boys) with mean age ± SD 4.9 ± 3.4 months, their diagnoses were galactosemia (n = 5), cytomegalovirus hepatitis (n = 7), tyrosinemia (n = 2), Alagille syndrome (n = 3), idiopathic neonatal hepatitis (n = 8), Niemann-Pick type C (n = 2), progressive familial intrahepatic cholestasis (n = 2), and arthrogryposis, renal dysfunction, cholestasis syndrome (n = 1).

Control group included 30 infants apparently healthy matching the patient groups for age and sex included (15 girls/15 boys) their mean age ± SD 6.3 ± 3.07 months. There was no statistically significant difference between studied groups regarding age at blood sampling ($P > 0.05$).

Jaundice, clay-colored stool, and ascites were the most presenting symptoms in both patients' groups (100%, 70%, 20% in BA group, respectively and 86.7%, 23.3%, 13.3 in non-BA group, respectively), whereas colored stool and abdominal distension were increased in non-BA group (76.7%, 40%, respectively) than BA group (30%, 33.3%, respectively). Hepatomegaly and splenomegaly in the BA group were present in 25 (83.3%) and 13 (43.3%) patients, respectively, whereas those in non-BA group were 23 (76.6%) and 14 (46.6%), respectively.

Serum AST and ALT levels were higher in the non-BA group, whereas gamma-glutamyl transferase, alkaline phosphatase,

TABLE 1. Hematological and liver function tests among the studied groups

Variable	Group I (biliary atresia) (n = 30) Mean ± SD	Range	Group II (nonbiliary atresia) (n = 30) Mean ± SD	Range	Group III (controls) (n = 30) Mean ± SD	Range	KW test	P
AST, U/L	252 ± 140*	38–679	299 ± 317*	32–1644	32 ± 5	22–41	55.5	<0.001 (HS)
ALT, U/L	208 ± 141*	17–540	215 ± 254*	13–1106	41 ± 10	29–59	37.8	<0.001 (HS)
GGT, U/L	707 ± 288*,†	297–1638	280 ± 290*	24–1500	35. ± 7	24–49	66.5	<0.001 (HS)
ALP, U/L	940 ± 389*,†	456–2380	345 ± 192*	112–914	89 ± 35	51–202	74.3	<0.001 (HS)
Total bilirubin, mg/dL	12.7 ± 11.15*	2.8–41.5	11.2 ± 8.67*	2.8–31.5	0.93 ± 0.12	0.7–1.2	59.5	<0.001 (HS)
Direct bilirubin, mg/dL	11.0 ± 8.73*	2.3–33.6	9.5 ± 6.16*	2.41–25.2	0.09 ± 0.04	0.02–0.16	59.4	<0.001 (HS)
Total protein, g/dL	5.73 ± 0.74*	4–7	5.62 ± 1.06*	3.8–8.1	7.04 ± 0.43	6–7.9	29.7‡	<0.001 (HS)
Albumin, g/dL	3.49 ± 0.68	2.1–4.6	3.60 ± 0.64	2.3–5.0	3.62 ± 0.14	3.31–3.8	0.46	0.62 (NS)
Hb, g/dL	10.6 ± 2.20*	6.9–17.4	9.4 ± 2.19*	5.7–14.7	12.9 ± 1.26	10.8–15.9	38.08	<0.001 (HS)
TLC, $\times 10^3/cm^3$	10.1 ± 3.9	2.7–19.1	14.6 ± 14.4*	2.55–83.0	8.3 ± 2.5	4.7–13.0	7.54	0.023 (S)
PLTs, $x10^3/cm^3$	267.3 ± 162.08	21–778	287.3 ± 184.88	25.5–687	319.1 ± 118.36	170–568	2.67	0.26 (NS)

ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma-glutamyl transferase, HB = hemoglobin, KW = Kruskal Wallis; PLTs = platelets, TLC = total leucocytic count.
*Sig in comparison with Gr III.
†Sig in comparison with Gr II, KW test → Kruskal-Wallis test.
‡Analysis of variance (ANOVA) test.

and bilirubin (total and direct) were higher in the BA group. Total protein was lower in non-BA group than the BA group and both had a lower level than the control group, whereas total leucocytic count was higher in non-BA group followed by BA group, and both were higher than thant in the control group (Table 1).

Regarding histopathological characteristics of patients' groups, mononuclear cells (plasma cells and lymphocytes) were present in 66.7% of BA group and 33.3% of non-BA group. Giant cells represent 33.3% of non-BA group and mean ± SD of fibrosis stage was 2 ± 0.8 in BA group, and 2.1 ± 0.9 in the non-BA group, mean ± SD of HAI was 5.6 ± 2.4 in BA group and 5.1 ± 2.1 in non-BA group, which was statistically nonsignificant (Table 2).

There was a significant difference between studied groups regarding serum IL-33 as it was higher in BA group (median 48.0 pg/mL, interquartile range [IQR]: 28.9–106.2) than non-BA group (median 17.3 pg/mL, IQR: 13.7–18.8) and both showed higher levels than control group (median 7.3 pg/mL, IQR: 5.8–9.99) (supplementary Fig. 1, Supplemental Digital Content, *http://links.lww.com/MPG/B745*).

There was a gradual increase in serum IL-33 level in accordance with fibrosis stage in BA group as the highest level in fibrosis stage *3/6* and the lowest level in fibrosis stage *1/6*; this is also the same in non-BA group as the highest level of IL-33 present in fibrosis stage 3/6 and the lowest level in fibrosis stage 0/6 stage. There was no statistically significant association between serum IL-33 and the Child-Pugh score (Table 3).

There was a positive correlation between serum IL-33 and each of AST, ALT, and bilirubin (total and direct) levels and fibrosis stage among the BA group. There was a positive correlation between serum IL-33 and bilirubin (total and direct) levels and

TABLE 2. Liver biopsy among biliary atresia group and nonbiliary atresia group

Variable	BA group (no = 30) No.	Percentage within groups	Non-BA group (no = 30) No.	Percentage within Groups	Fisher exact test	P
Type of cells						
Lymphocytes	10	33.3	8	26.7	3.7	0.49 (NS)
Eosinophils	3	10.0	5	16.7		
Giant cells	0	0.0	10	33.3		
Mononuclear cells (plasma cells and lymphocytes)	20	66.7	10	33.3		
Fibrosis stage						
0/6	0	0.0	3	10.0	5.93	0.092 (NS)
1/6	9	30.0	3	10.0		
2/6	11	36.7	11	36.7		
3/6	10	33.3	13	43.3		
Mean ± SD	2 ± 0.8		2.1 ± 0.9		$Z_{MWU} = 0.75$	0.45 (NS)
Range	1–3		0–3			
HAI						
Mean ± SD	5.6 ± 2.4		5.1 ± 2.1		$Z_{MWU} = 1.38$	0.16 (NS)
Range	1–8		2–9			

BA = biliary atresia; SD = standard deviation.

TABLE 3. Comparison between serum interleukin-33 and both fibrosis stage and child Pugh score in patients' group

Variable	No	Serum IL-33			KW test	*P*
		Mean	± SD	Range		
Biliary atresia group						
Fibrosis stage						
1/6*	9	37.2	35.3	20.6–131.4	11.6	0.001 (HS)
2/6	11	70.9	53.3	29.9–155.5		
3/6*	10	88.2	37.1	50.6–146.7		
Child-Pugh score						
A	6	64.7	45.5	24.8–131.4	0.62	0.73 (NS)
B	19	62.0	44.9	20.6–154.7		
C	5	86.0	60.1	26.4–155.5		
Nonbiliary atresia group						
Fibrosis stage						
0/6*,†	3	8.6	4.33	3.6–11.2	11.6	0.009 (S)
1/6	3	14.7	5.48	11.5–221.1		
2/6*	11	15.8	2.58	13–19.9		
3/6†	13	18.5	1.83	16.4–22.3		
Child-Pugh score						
A	8	15.7	3.11	11.2–20.0	0.89	0.64 (NS)
B	18	16.4	4.59	3.6–22.3		
C	4	16.1	3.00	11.7–18.3		

KW = Kruskal Wallis; IL-33 = Interleukin-33; SD = standard deviation.
*Sig in comparison with Gr F2.
†Sig in comparison with F3.

fibrosis stage among the non-BA group. There was no statistically significant correlation between age and IL-33 in both studied groups irrespective of some cases with BA diagnosed at a later age (Table 4).

Serum IL-33 at a cut-off value of ≥20.8 pg/mL can detect BA with a specificity of 95% and a sensitivity of 96.7%, PPV 90.6%, NPV 98.3%, AUC 0.995 ($P < 0.001$). While at a cut-off value of ≥45.3 pg/mL, IL-33 can detect liver fibrosis of significant fibrosis (F3) with a specificity of 72.2% and a sensitivity of 66.7% PPV 54.5%, NPV 81.2%, AUC 0.8 ($P < 0.03$). (Supplementary Fig. 2A and B, Supplemental Digital Content, *http://links.lww.com/MPG/B745*)

Factors found to be significantly correlated with IL-33 were entered the multiple linear regression analysis models to detect the significant predictors of IL-33 level. It was found that AST and direct bilirubin are the significant predictors ($P < 0.05$ for both). Also, binary logistic regression detects that (IL 33 ≥20.8) compared with those without can predict BA among patients with neonatal cholestasis (Supplementary Table 1, Supplemental Digital Content, *http://links.lww.com/MPG/B745*).

DISCUSSION

The inflammatory process in BA is an ongoing one leading to progressive scarring of the liver and severe fibrosis ending in cirrhosis even after successful surgery. This is possibly due to the tenacious release of proinflammatory cytokine by immune cells in the portal tracts around bile duct (15). The induction and maintenance of systemic and local inflammatory responses play a pivotal role in this process. The expression of proinflammatory cytokines induces the expression of adhesion molecules and the production of chemokines by endothelial cells, which encourages the recruitment of inflammatory cells and perpetuates liver damage (16).

In the present study, liver biopsy among the studied patients shows mononuclear cells were 66.7% in BA group and 33.3% in the non-BA group, giant cells represent 33.3% in non-BA group and fibrosis stage in BA group was mean ± SD = 2 ± 0.8 and 2.1 ± 0.9 in non-BA group, mean ± SD of HAI was 5.6 ± 2.4 in BA group and 5.1 ± 2.1 in non-BA group, which was statistically nonsignificant.

Arafa et al (17) found that higher grades of portal cellular infiltrate (moderate/severe) were frequently observed in the BA group (45%), whereas most of the non-BA group had either no/minimal (35.7%) or mild (39.3%) portal cellular infiltrate. The degree of activity reflects lymphocytic and neutrophilic infiltration. Mack et al (18) and Pacheco et al (19) described a prominent portal inflammation in BA. Shaalan et al (20) found that parenchymal changes such as multinucleated giant cell transformation and hepatocyte swelling were found to be significantly higher in the BA group than in the NC group. On the contrary, Lee and Loi (21) reported that lymphocytic and neutrophilic infiltrations were uncommon in BA ranging between 3% and 20%.

In the present study, there was a significant difference between studied groups regarding serum IL-33 as it was higher in the BA group than in the non-BA group than in the control group. These results run in accordance with Dong et al (22), who studied Chinese infants and divided them to BA group and choledochal cyst (CC) group with control, they found that the mean concentration of serum IL-33 was significantly elevated in BA infants, as compared with the CC and healthy control groups. Dong et al, however, compared BA to only nonicteric children, but in our study and for clinical decision process it was important to compare patients with BA to a group of icteric children due to other diseases. Therefore, IL 33 seems to be reliable, noninvasive, nonexpensive, and fast tool compared for other markers for detecting patients with BA.

TABLE 4. Correlation between serum interleukin-33 and clinical and laboratory data among patients' groups

	Serum IL-33			
	BA group (n = 30)		Non-BA Group (n = 30)	
Variable	Rho	*P*	Rho	*P*
Age, mo	0.013	0.94	−0.286	0.12
Weight, kg	−0.079	0.67	−0.134	0.48
Height, cm	0.113	0.55	−0.127	0.51
HC, cm	0.170	0.37	−0.112	0.56
BMI, kg/m^2	−0.278	0.13	−0.077	0.68
Liver span, cm	−0.155	0.41	−0.304	0.10
Spleen size, cm	−0.174	0.35	−0.085	0.65
Fibrosis stage	0.658	<0.001 (HS)	0.568	0.001 (HS)
HAI	0.035	0.85	0.286	0.12
PELD score	0.100	0.59	0.011	0.95
AST, U/L	0.490	0.006 (S)	0.215	0.25
ALT, U/L	0.396	0.03 (S)	0.230	0.22
GGT, U/L	0.139	0.46	0.193	0.31
ALP, U/L	0.01	0.95	0.07	0.71
T. bilirubin, mg/dL	0.367	0.046 (S)	0.385	0.036 (S)
D. bilirubin, mg/dL	0.393	0.032 (S)	0.363	0.049 (S)
T. protein, g/dL	0.154	0.41	−0.067	0.72
Albumin, g/dL	0.057	0.76	−0.079	0.68
Hb, g/dL	−0.036	0.84	−0.104	0.58
TLC, $\times 10^3$/cm^3	0.108	0.57	−0.032	0.86
PLTs, $\times 10^3$/cm^3	0.113	0.55	0.077	0.68

ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BA = biliary atresia; GGT = gamma-glutamyl transferase; HB = hemoglobin; IL-33 = Interleukin-33; PLTs = platelets; TLC = total leucocytic count.

Also, McHedlidze et al demonstrated significantly higher serum amounts of IL-33 that were observed in patients compared to controls, also IL-33 was overexpressed in hepatocytes of IL-33 vector-injected but not control vector-injected mice and IL-33 protein was detected in serum. As IL-33 is released in response to chronic hepatocellular stress and that extracellular IL-33, via ST2-dependent signaling, leads to accumulation and activation of ILC2 in the liver. Activated hepatic ILC2 produce IL-13, which in turn triggers activation and transdifferentiation of HSCs in an IL-4Rα- and STAT6 transcription factor-dependent fashion. These findings characterize molecular and cellular networks implicated in hepatic fibrosis and highlight the role of IL-33 at the apex of the profibrotic cascade (23,24).

Also, IL-33 has the capacity to increase the production of inflammatory cytokines only in injured livers (25,26). IL-33 signals through a unique IL-1 receptor-related protein that is named ST2, also called IL-33R. The binding of IL-33 to ST2 activates nuclear factor-κB and mitogen-activated protein kinases and drives the production of proinflammatory and T helper type 2 (Th2)-associated cytokines (27).

In the present study, the best cut-off of serum IL-33 was 20.8 pg/mL to diagnose BA with specificity 95% and sensitivity 96.7% ($P < 0.05$), also, serum IL-33 at a cut-off value of 45.3 pg/mL can predict liver fibrosis of significant fibrosis (F3) with specificity 72.2% and sensitivity 66.7% ($P < 0.05$). Chen and Qian's (28), study on infants with CMV found that IL-33 had 95% sensitivity and 68.7% specificity at a cut-off value of 2.04 pg/mL to predict liver fibrosis in infants with CMV.

Oztas et al (29) reported that the cutoff value of serum IL-33 was taken as >1207.7 pg/mL for diagnosis of significant fibrosis (F4) with a sensitivity of 52.94% and specificity of 56.86% ($P = 0.834$). IL-33 serves as an ''alarmin'' released by stressed hepatocytes. IL-33 then attracts ILC2, which trigger the profibrogenic activation of HSCs via mediators such as IL-13 (8). The value of IL-33 as fibrosis marker may, however, be with little value because it is influenced by many other circumstances/diseases.

Strength and limitations: serum IL33 could be better than other diagnostic markers of BA because it is an early noninvasive, nonexpensive, and fast tool. Limitations in our study include small number of cases.

CONCLUSION

The significantly higher production of IL-33 in patients with BA compared to non-BA suggests a potential role of IL-33 for initiation and progression of the disease process. IL-33 may have a diagnostic value in infants with BA.

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