Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

T cell cytokines in the diagnostic of early-onset sepsis

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BACKGROUND: Early-onset sepsis (EOS) remains a substantial cause of morbidity and mortality among neonates. Yet, currently available biological parameters have not proven to be accurate enough to predict EOS reliably. This study aimed to determine serum concentrations of 13 cytokines in umbilical cord blood and evaluate their diagnostic value for EOS.

METHODS: A prospective single-center study that included analysis of umbilical cord blood of term and preterm neonates who were born from March 2017 to November 2017. Using ELISA analysis, 13 cytokines were simultaneously quantified and correlated with the development of EOS.

RESULTS: Four hundred and seventy-four neonates were included, of which seven met the criteria for culture-positive EOS. Interleukin (IL)-6 ($p < 0.001$), IL-9 ($p = 0.003$), and IL-21 ($p < 0.001$) were significantly increased in neonates with EOS compared to controls. Sensitivity and specificity for IL-6, IL-9, and IL-21 at the defined cut-off points were 85.7 and 77.3%, 71.4 and 62.5%, and 71.4 and 52.0%, respectively.

CONCLUSIONS: In neonates with EOS, IL-9 and IL-21 are significantly elevated and may be employed in the diagnostic of EOS. However, diagnostic accuracy remains lower than with IL-6. Values of 13 T cell cytokines may be used as reference values for future studies in neonates.

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IMPACT:

- Interleukin-9 (IL-9) and interleukin-21 (IL-21) are significantly elevated in neonates with early-onset sepsis.
- IL-9 and IL-21 have been shown to play a specific role in neonatal sepsis.
- Neonatal reference values were generated for several cytokines.
- IL-9 and IL-21 might be attractive biomarkers for neonatal sepsis in future. This study is likely to promote further research in this area.
- Values of several T cell cytokines may be used as reference values for future studies in neonates.

INTRODUCTION

Despite advances in neonatal management, early-onset neonatal sepsis (EOS) remains a significant cause of morbidity and mortality both among term and preterm infants.[1] The outcome and prognosis of EOS depend substantially on early and efficient treatment. Still, accurate diagnosis remains a major challenge as clinical signs are non-specific and conventional biomarkers reveal limited diagnostic sensitivity and specificity to predict EOS reliably.[2,3] As a consequence, empirical antibiotic treatment is frequently applied in neonates, causing unnecessary exposure to adverse effects and the promotion of antimicrobial resistance.[4]

Over the past two decades, cytokines have received increasing scientific attention in the diagnosis of sepsis. Cytokines are small peptides that play a central role in inflammatory processes and immune response and comprise chemokines, interleukins (ILs), interferons (IFNs), lymphokines, and tumor necrosis factors (TNFs).[5–7] They are secreted by a variety of cell types and can rapidly increase their concentrations from picograms to micrograms per milliliter during sepsis.[5,6,8] Because acute-phase reactants such as C-reactive protein (CRP) are produced in response to proinflammatory cytokines, direct measurement of serum cytokine levels has the potential to provide an earlier indication of infection.

IL-6 is the most studied cytokine in neonates. It is a multifunctional proinflammatory cytokine that has shown to be an early and highly sensitive marker of bacterial infection in neonates. Yet it has also been demonstrated to be poorly specific in critically ill infants.[9–12] Other cytokines that are potentially useful for early diagnosis of neonatal infections are IL-Iβ, IL-8, IL-10, transforming growth factor β, soluble IL-2 receptor, and TNF-α.[13,14] However, the cytokine family has kept growing over the past years, and numerous previously surfaced cytokines have not been investigated in the context of neonatal sepsis.

This study analyses and evaluates the value of 13 human cytokines, which are collectively secreted by T helper cells for early and non-invasive diagnosis of EOS using umbilical cord blood.

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METHODS

Study design and subjects

We conducted a prospective study at our tertiary perinatal center. Our population consisted of term and preterm neonates, who were recruited at birth from March to November 2017. Mother–infant pairs were eligible for enrollment if a cord blood sample was taken immediately after birth, and parental consent was obtained.

Patients were divided into three groups: (1) neonates diagnosed with EOS based on clinical signs within the first 72 h of life and confirmed by positive blood culture (culture-positive sepsis); (2) neonates with suspected sepsis with a CRP elevation ≥10 mg/l within the first 72 h of life together with the presence of two or more of the following clinical signs: temperature instability, respiratory symptoms, cardiovascular symptoms, neurological symptoms, or abdominal symptoms (culture-negative sepsis); and (3) neonates with no signs of EOS. Preterm neonates with a gestational age (GA) <32 weeks were excluded from the study as most infants in this patient group received prophylactic antibiotic treatment as a result of our standard protocol.

Clinical patient data were obtained prospectively from the digital medical chart (Soarian®, Siemens Healthcare, Erlangen, Germany).

The study protocol was approved by the ethics committee of the local medical chamber of Hamburg.

Sample collection and analysis

Umbilical cord blood samples were collected immediately after birth. CRP was directly analyzed in the medical laboratory of our hospital using particle-enhanced immunonephelometry (Dimension Vista 1500, Siemens Healthcare, Erlangen, Germany). Plasma was stored at −80 °C until cytokine analysis. The human T helper cytokines were measured by flow cytometry using LEGENDplex™ assay according the manufacturer's protocol (Human Th Panel (13-plex), BioLegend, San Diego, CA). Analysis of quantification of the 13 human cytokines IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, IFN-γ, and TNF-α was performed with the GraphPad Prism software (GraphPad, San Diego, CA).

Statistical analysis

All data were analyzed using SPSS Statistics 25 (IBM, New York, NY) and GraphPad Prism 8 (GraphPad, San Diego, CA). Data on patient demographics are expressed as median and range for continuous variables and as counts and category percentages for categorical variables. Comparison of demographic data between neonates with and without infection was conducted by independent *T* test for continuous parameters and categorical variables, and the risk for infection was analyzed by Pearson's χ^2 test for dichotomous parameters and by Mann–Whitney *U* tests for ordinal parameters.

Box and whisker plots were applied to compare the three different groups visually. The 2.5 and 97.5 percentiles of the cytokine values were calculated to establish the upper and lower limits of the reference interval.

The strength of associations between cytokine levels and EOS was determined using non-parametric Mann–Whitney *U* test. Two-tailed p value < 0.05 was considered significant. The Holm–Bonferroni method was applied to adjust the statistical inference of multiple comparisons.

Receiver operating characteristic (ROC) curves and area under the curve (AUC) were calculated to analyze the performance of the significant cytokines in the diagnosis of neonatal infection. Sensitivity, specificity, and the Youden Index were generated and applied to determine the optimal cut-off values in the ROC analysis.

RESULTS

Demographics

During the observational period, there were 2951 deliveries with newborns >32 weeks of gestational age. Cord blood samples, parental consent, complete data collection, and sample analysis were obtained from 474 neonates, of which 7 neonates met the defined criteria for culture-positive EOS. Ten infants with a negative blood culture presented with clinical signs of sepsis. Peripartum characteristics were similar in the groups with and without EOS (Table 1). Clinical sepsis signs and blood culture results for all patients treated for sepsis are presented in Table 2.

Mean cytokine concentrations and standard deviations for neonates with and without EOS are shown in Table 3. Bivariate analysis using Mann–Whitney *U* test revealed significant differences between the groups for IL-6, IL-9, and IL-21. Analysis revealed no significant differences of cytokine levels between sepsis patients with and without positive blood culture.

Differences of cytokine concentrations between neonates with and without sepsis are visualized with box and whisker plots in Fig. 1.

Diagnostic accuracy of IL-6, IL-9, and IL-21

In ROC analysis, AUC was 0.923 for IL-6, 0.772 for IL-9, and 0.739 for IL-21 when EOS was defined as culture-positive sepsis only. Using ROC and postulating a minimum test sensitivity of 70%, we defined cut-off points for IL-6, IL-9, and IL-21 at 45, 7.0, and 10 pg/ml,

Table 1. Characteristics of the 474 neonates included in the study.

Characteristics	No infection ($n = 457$)	Culture-positive sepsis ($n = 7$)	Culture-negative clinical sepsis ($n = 10$)	p value
Maternal characteristics				
Age, years	33.3 (18.5–46.3)	34.0 (18.8–41.0)	35.1 (26.8–42.7)	0.718[a]
GBS carriage status positive, *n* (%)	91 (20)	2 (29)	4 (40)	0.164[b]
ROM, h	7.4 (0–68.1)	4.1 (0–10.2)	6.1 (0.1–42.9)	0.672[a]
C-section, *n* (%)	111 (24)	0 (0)	1 (10)	0.170[b]
Child characteristics				
Female, *n* (%)	225 (49)	4 (57)	5 (50)	0.917[b]
Birth weight, g	3390 (1350–4930)	3270 (2338–4025)	3595 (2660–4850)	0.302[a]
Gestational age, weeks	39.6 (32.9–42.3)	40.1 (35.1–41.0)	40.1 (36.7–41.9)	0.411[a]

Values are given as median (range) unless stated otherwise. GBS group B streptococcus, ROM rupture of membranes.
GBS group B *Streptococcus*, *ROM* rupture of membranes.
[a]Independent *T* test.
[b]Pearson's χ^2 test.

Table 2. Clinical signs and blood culture in 17 neonates with proven or suspected sepsis.

Patient	GA (weeks + days)	Birth weight (g)	Clinical signs of sepsis	Blood culture
1	40 + 4	4025	Respiratory distress, poor perfusion	*Streptococcus agalactiae*
2	39 + 6	2895	Hypothermia, hypoglycemic, lethargy	*Escherichia coli*
3	39 + 2	4850	Hypothermia, hypoglycemic, lethargy	*Enterobacter species*
4	41 + 4	4220	Poor perfusion, hypothermia	*Escherichia coli*
5	34 + 6	2338	Respiratory distress, hypotonia, lethargy	*Escherichia coli*
6	34 + 6	2720	Respiratory distress, poor perfusion, lethargy	*Streptococcus viridans*
7	40 + 1	3500	Respiratory distress, poor perfusion, lethargy, fever	*Streptococcus agalactiae*
8	40 + 1	3690	Fever, lethargy	Negative
9	39 + 3	3715	Respiratory distress, hypothermia	Negative
10	38 + 5	2660	Poor perfusion, hypothermia	Negative
11	36 + 5	2920	Fever, lethargy	Negative
12	41 + 0	4400	Respiratory distress, poor perfusion, fever	Negative
13	40 + 2	3815	Respiratory distress, poor perfusion	Negative
14	41 + 0	3180	Respiratory distress, poor perfusion, bradycardia	Negative
15	41 + 6	3475	Poor perfusion, hypothermia, lethargy	Negative
16	40 + 1	3270	Respiratory distress, lethargy	Negative
17	40 + 1	3250	Poor perfusion, hypothermia	Negative

GA gestational age.

Table 3. Associations between cytokine levels and EOS.

Cytokines (pg/ml)	No infection ($n = 457$)	Culture-positive sepsis ($n = 7$)	Culture-negative clinical sepsis ($n = 10$)	*p* value			Adjusted *p* value[a]		
				NI-CPS	NI-CNS	CPS-CNS	NI-CPS	NI-CNS	CPS-CNS
IL-2	12.3 (26.0)	7.3 (3.2)	6.3 (1.8)	0.608	0.465	0.442	1.000	1.000	1.000
IL-4	0.6 (4.7)	0.0 (0.0)	0.0 (0.0)	0.724	0.673	[b]	1.000	1.000	[b]
IL-5	11.9 (5.5)	11.8 (4.9)	12.1 (1.0)	0.990	0.909	0.890	1.000	1.000	1.000
IL-6	35.6 (42.2)	296.6 (370.0)	419.9 (861.8)	<0.001	<0.001	0.728	<0.001	<0.001	1.000
IL-9	6.7 (4.7)	10.6 (4.7)	9.7 (7.6)	0.003	0.005	0.776	0.034	0.052	1.000
IL-10	3.5 (7.8)	5.6 (5.1)	7.0 (8.4)	0.478	0.166	0.707	1.000	1.000	1.000
IL-13	24.1 (0.9)	24.0 (0.5)	24.1 (0.5)	0.668	0.997	0.572	1.000	1.000	1.000
IL-17A	0.4 (2.0)	0.0 (0.0)	0.0 (0.0)	0.572	0.479	0.244	1.000	1.000	1.000
IL-17F	28.1 (1.3)	28.7 (2.1)	28.4 (1.1)	0.201	0.366	0.735	1.000	1.000	1.000
IL-21	12.7 (12.3)	26.1 (19.2)	21.3 (16.9)	<0.001	<0.001	0.591	0.013	0.006	1.000
IL-22	10.1 (6.6)	4.8 (5.9)	11.1 (4.1)	0.134	0.618	0.019	1.000	1.000	0.253
IFN-γ	13.9 (23.8)	14.1 (6.2)	14.1 (4.0)	0.975	0.977	0.979	1.000	1.000	1.000
TNF-α	2.5 (7.3)	2.3 (4.2)	0.6 (1.1)	0.968	0.434	0.242	1.000	1.000	1.000

Values are shown as mean (SD).
NI no infection, *CPS* culture-positive sepsis, *CNS* culture-negative sepsis, *IL* interleukin, *IFN* interferon, *TNF* tumor necrosis factor.
[a]*p* value adjusted using Holm–Bonferroni method.
[b]*p* value cannot be calculated because the standard deviations of both groups are 0.

respectively. Sensitivity and specificity for the three cytokines alone and for both IL-9 and IL-21 in combination with IL-6 are displayed in Table 4. Higher sensitivity was obtained when combining IL-9 or IL-21 with IL-6, and an infection was assumed if at least one of the cytokines were positive. But it also caused a decrease of specificity as a result of an increase in false-positive cases.

Cytokine reference levels

Cord plasma reference values were generated for all the analyzed cytokines in the 457 neonates without clinical signs of neonatal infection. Calculated median and range values, as well as 2.5 and 97.5 percentiles, are shown in Table 5. Regression analysis revealed no association between cytokine levels and gestational age.

DISCUSSION

In this study, we evaluated the diagnostic value of 13 T cell cytokines, of which, to date, several have not been investigated in the context of neonatal sepsis. We detected significantly higher cord blood values of IL-6, IL-9, and IL-21 in neonates with infection compared to healthy neonates.

IL-6 has been intensively studied and is known to be an early and highly sensitive marker for neonatal sepsis.[9–12]

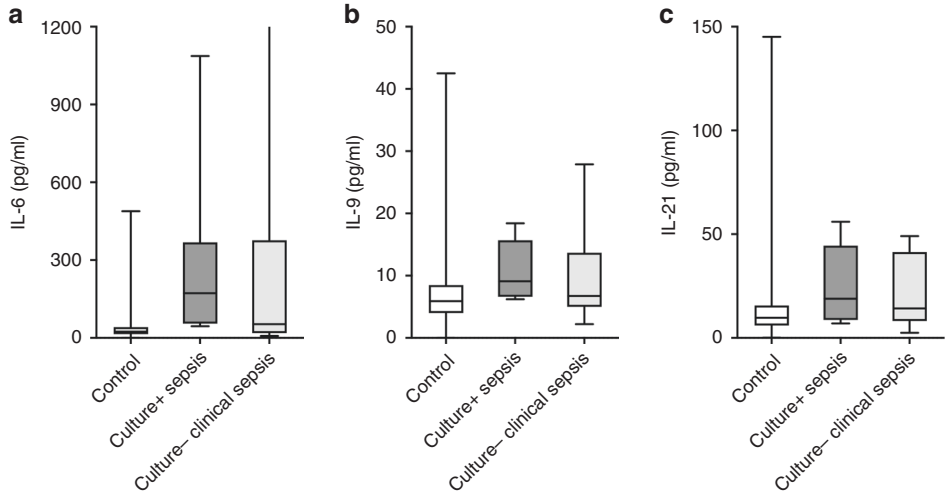

Fig. 1 Box and whisker plots of IL-6 (a), IL-9 (b), and IL-21 (c). Values are showing median, interquartile range, and min/max of neonates with and without infection.

Table 4. Test characteristics of IL-6, IL-9, and IL-21 alone and in combination for early-onset neonatal sepsis.

Cytokine	EOS defined as CPS only		EOS defined as CPS or CNS	
	Sensitivity	Specificity	Sensitivity	Specificity
IL-6	85.7	77.3	76.5	78.1
IL-9	71.4	62.5	58.8	62.8
IL-21	71.4	52.0	70.6	52.5
IL-6/IL-9	85.7	54.0	82.4	54.7
IL-6/IL-21	85.7	48.4	76.5	48.8

Values are percentages.
EOS early-onset neonatal sepsis, *CPS* culture-positive sepsis, *CNS* culture-negative sepsis, *IL* interleukin.

Table 5. Cytokine reference values (pg/ml) in the cord blood plasma of 457 neonates with no clinical signs of infection.

Cytokine	Median (range)	2.5th perc.	97.5th perc.
IL-2	5.8 (1.9–233.5)	3.3	90.0
IL-4	0.0 (0.0–57.9)	0.0	3.0
IL-5	10.8 (0.0–56.3)	4.6	25.4
IL-6	24.5 (0.0–488.5)	5.4	128.0
IL-9	5.9 (0.0–42.5)	0.3	17.2
IL-10	1.7 (0.0–101.7)	0.0	17.0
IL-13	24.0 (22.2–33.6)	22.8	26.1
IL-17A	0.0 (0.0–21.1)	0.0	4.3
IL-17F	28.0 (25.6–37.3)	26.2	31.0
IL-21	9.6 (0.0–145.0)	0.9	42.5
IL-22	11.3 (0.0–55.5)	0.0	22.0
IFN-γ	11.8 (0.0–503.7)	4.7	27.5
TNF-α	0.0 (0.0–94.5)	0.0	19.0

IL interleukin, *IFN* interferon, *TNF* tumor necrosis factor, *perc.* percentile.

The concordance of our findings with established evidence demonstrates the representative character of our study cohort for the analysis of cytokines for EOS. The sensitivity and specificity for IL-6 in the diagnosis of EOS in this study were 86 and 77%, respectively, when EOS was defined as culture-positive patients only. In previous literature evaluating the diagnostic accuracy of IL-6 in neonatal sepsis, the wide variation in sensitivity (53–90%) and specificity (44–100%) is presumably caused by the heterogeneity of study designs, including varying definitions of cut-off values.[9–11,15–18]

IL-9 and IL-21 are pleiotropic cytokines that play a crucial role in inflammation and infection.[19] IL-9 has been most frequently associated with allergic inflammation and immunity to extracellular parasites.[20] But there is also evidence that IL-9 plays a role in the regulation of immunity to infectious disease via enhancement of IL-4-mediated IgE and IgG production from human B cells.[21,22] IL-21 is a critical component of T cell-directed B cell activation, proliferation, and class switch recombination and is therefore a potent immune-modulatory cytokine playing a key role in infection.[19,23] The fact that both IL-9 and IL-21 were elevated indicates a specific function of Th9 cells in neonatal sepsis as these CD4+ cells have been shown to produce IL-9 and IL-21.[24] Also, IL-21 promotes Th9 cells to secrete IL-9, thus forming a positive feedback on its differentiation and function.[25] Whereas no studies exist for IL-9 in the context of neonatal sepsis, IL-21 levels have been previously demonstrated to be elevated in the peripheral blood of septic neonates.[26] In our study, although IL-9 and IL-21 showed significantly higher mean levels in infected neonates compared to controls, diagnostic accuracy was inferior to IL-6. At the defined cut-off point that generated a moderate sensitivity of 71%, the specificity was only 63 and 52% in IL-9 and IL-21, respectively. A higher sensitivity was obtained when combining IL-9 or IL-21 with IL-6 and infection was assumed if either one of the cytokines was positive. However, as there is typically a trade-off between sensitivity and specificity, a combination resulted in a marked decrease of specificity to a range of 54 and 48%, respectively.

Most cytokines in our study revealed no significant elevation in the cord blood of neonates with EOS. Yet, some of these cytokines like TNF-α, IL-4, and IL-10 have previously been shown to be elevated in neonatal sepsis. Previous study results evaluating TNF-α have been contradictious as both significant elevations and no differences of TNF-α levels have been detected in septic newborns compared to healthy neonates.[14,27,28] The discrepancy between the studies might be explained with different study designs as well as the not fully understood kinetics of TNF-α. Anti-inflammatory cytokines, such as IL-4 and IL-10, play an important

role in the prevention of excess proinflammatory response during sepsis and strive to restore immunological balance.[29] Khaertynov et al. observed that the production of IL-4 and IL-10 became substantially upregulated only during the post-acute phase and that they were increased in patients with late-onset sepsis but not EOS, which conforms to our observation that these two cytokines were not increased in our neonates with EOS.[30] Other cytokines that were not elevated in neonates with sepsis in our study were IL-13, IL-17A, IL-17F, IL-22, and INF-γ. Consistent with our research, Sugitharini et al. previously demonstrated that the anti-inflammatory cytokine IL-13 was not elevated in septic neonates.[26] As members of the IL-17 family, the cytokines IL-17A and IL-17F promote inflammation and have been associated with allergic responses and the pathogenesis of various autoimmune-related diseases, such as psoriasis.[31] Ahmed Ali et al. also found that IL-17 was significantly associated with sepsis in polytrauma patients.[32] Still, up to date, there has been no study investigating IL-17 in neonatal infection. IL-22 is produced by several populations of immune cells at a site of inflammation and plays an essential role in the stimulation of cell survival, proliferation, and synthesis of antimicrobials.[33] Although animal studies suggest that IL-22 may play a crucial role in clinical sepsis,[34,35] little is known about IL-22 in sepsis patients. One single-center study reports an elevation of serum IL-22 in abdominal sepsis patients.[36] IFN-γ is a potent activator of macrophages and a signature cytokine of activated T lymphocytes. It is, therefore, critical for the innate and adaptive immunity against viral, bacterial, and protozoal infections.[37–39] The few studies that have investigated IFN-γ in neonates report inconsistent results regarding its levels during sepsis.[40,41]

Statistical analysis revealed no significant differences in cytokine levels between patients with culture-confirmed EOS and those with clinical signs of sepsis and negative blood culture. An explanation could be either the actual presence of an infection with falsely negative blood culture or a clinically significant systemic inflammatory response due to non-infectious sources in the latter group's patients.[42,43]

Over the past decades, cytokines have been identified as promising biomarkers in the diagnostic and as a predictor in several diseases in adults such as infections,[44–46] chronic diseases,[31,47,48] and obstetric and gynecological conditions.[49,50] As further indications for cytokine measurements might occur in the future, one aim of the present study was to establish normal cytokine values in neonates. The reference ranges of 13 simultaneously measured T cell cytokines generated via analysis of a substantial cohort of neonates might be of great value for future studies and clinical diagnostics.

CONCLUSION

The proinflammatory cytokines IL-9 and IL-21 are significantly elevated in the cord blood of neonates with EOS. However, diagnostic accuracy remains lower than with IL-6. Thus further investigations are necessary to evaluate the diagnostic value of these and other cytokines in neonatal infection. We formulated recommendations for reference values for various T cell cytokines in neonates based on a large cohort.

AUTHOR CONTRIBUTIONS

G.M.F. assisted in conceptualizing the study, carried out statistical analyses, interpreted data, and drafted the initial manuscript. T.B. performed laboratory analysis of the cord blood, interpreted data, and assisted in the drafting of the manuscript. M.B. interpreted the data and assisted in the drafting of the manuscript. S.H. assisted in conceptualizing the study and interpreted the data. D.S. assisted in conceptualizing the study and interpreted the data. C.U.E. conceptualized and designed the study, collected data, carried out statistical analyses, interpreted data, and drafted the initial manuscript. All authors reviewed and revised the manuscript, approved the final manuscript as submitted, and agree to be accountable for all aspects of the work.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

Patient consent: Patient consent was not required.

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