

Cytokine profiles of preterm neonates with fungal and bacterial sepsis

Beena G. Sood¹, Seetha Shankaran¹, Robert L. Schelonka², Shampa Saha³, Danny K. Benjamin Jr⁴, Pablo J. Sánchez⁵, Ira Adams-Chapman⁶, Barbara J. Stoll⁶, Poul Thorsen⁷, Kristin Skogstrand⁸, Richard A. Ehrenkranz⁹, David M. Hougaard³, Ronald N. Goldberg⁴, Jon E. Tyson¹⁰, Abhik Das¹¹, Rosemary D. Higgins¹², Waldemar A. Carlo² for the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network

BACKGROUND: Information on cytokine profiles in fungal sepsis (FS), an important cause of mortality in extremely low birthweight (ELBW) infants, is lacking. We hypothesized that cytokine profiles in the first 21 d of life in ELBW infants with FS differ from those with bacterial sepsis (BS) or no sepsis (NS).

METHODS: In a secondary analysis of the National Institute of Child Health and Human Development Cytokine study, three groups were defined—FS (≥ 1 episode of FS), BS (≥ 1 episode of BS without FS), and NS. Association between 11 cytokines assayed in dried blood spots obtained on days 0–1, 3 ± 1 , 7 ± 2 , 14 ± 3 , and 21 ± 3 and sepsis group was explored.

RESULTS: Of 1,066 infants, 89 had FS and 368 had BS. As compared with BS, FS was more likely to be associated with lower birthweight, vaginal delivery, patent ductus arteriosus, postnatal steroids, multiple central lines, longer respiratory support and hospital stay, and higher mortality ($P < 0.05$). Analyses controlling for covariates showed significant group differences over time for interferon- γ (IFN- γ), interleukin (IL)-10, IL-18, transforming growth factor- β (TGF- β), and tumor necrosis factor- α (TNF- α) ($P < 0.05$).

CONCLUSION: Significant differences in profiles for IFN- γ , IL-10, IL-18, TGF- β , and TNF- α in FS, BS, or NS in this hypothesis-generating secondary study require validation in rigorously designed prospective studies and may have implications for diagnosis and treatment.

Although advances in neonatal intensive care have led to improved survival, sepsis continues to be a major cause of mortality in extremely low birthweight (ELBW) infants (1). Fungal sepsis (FS) is particularly problematic because of difficulty in diagnosis and poor prognosis among infected infants. As compared with infants with bacterial sepsis (BS), infants with FS have a higher mortality rate and higher incidences of severe retinopathy of prematurity, periventricular leukomalacia, bronchopulmonary dysplasia, and significant

neurodevelopmental impairment (57%), resulting in increased length of stay and hospital costs of \sim \$30,000,000/y in the United States (2–4).

ELBW infants are at risk for invasive FS because of an immature protective layer of skin, the need for invasive medical devices, and defects in cellular and humoral responses (1). A definitive diagnosis, based on blood, cerebrospinal fluid (CSF), or urine culture, is usually reached only after a delay of 2–3 d (5). Rapid progression of an untreated infection may greatly increase morbidity and mortality. Although cytokine levels and acute-phase reactants have been used as markers of neonatal sepsis in general, the response of these biomarkers to FS has not been studied.

The first step in the innate immune response to fungal infections is recognition of fungal polymers (β -1, 3/ β -1, 6 glucans, glucuronoxylomannan, phospholipomannan, and galactomannan) by innate immune effector cells (6). In animal models and human adults, adaptive antifungal immunity includes T helper (Th)1-biased responses, which are protective, and Th2-biased responses, which are maladaptive or deleterious (7). Although CD4⁺ T cells have been classically divided as either Th1 or Th2, a new T helper effector subset, Th17, has been reported to play a seminal role in antifungal defense (8–13). Knowledge of the cytokine response in ELBW infants with and without FS is important to corroborate the information from animal models and human adults, to understand immune regulation and host defense in these infants, and to develop novel methods for diagnosis and treatment of FS.

The primary objective of this study was to assess cytokine levels and their temporal trends in dried blood spots (DBSs) collected over the first 3 wk after birth in ELBW infants with FS, BS, or no sepsis (NS). Eleven cytokines identified as being important in the development of FS were investigated: (i) Th-1 cytokines and their inducers, tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-12, IL-18, interferon- γ (IFN- γ);

¹Department of Pediatrics, Wayne State University, Detroit, Michigan; ²Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama; ³Statistics and Epidemiology Unit, RTI International, Research Triangle Park, North Carolina; ⁴Department of Pediatrics, Duke University, Durham, North Carolina; ⁵Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas; ⁶Department of Pediatrics, Emory University School of Medicine, Children's Healthcare of Atlanta, Atlanta, Georgia; ⁷Department of Obstetrics and Gynecology, Lillebaelt Hospital, Kolding, Denmark; ⁸Section of Neonatal Screening and Hormones, Department of Clinical Biochemistry and Immunology, Statens Serum Institut, Copenhagen, Denmark; ⁹Department of Pediatrics, Yale University School of Medicine, New Haven, Connecticut; ¹⁰Department of Pediatrics, University of Texas Medical School at Houston, Houston, Texas; ¹¹Statistics and Epidemiology Unit, RTI International, Rockville, Maryland; ¹²Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland. Correspondence: Beena G. Sood (bsood@med.wayne.edu)

(ii) Th-2 cytokines and their inducers, IL-4, IL-5, IL-6, IL-10; and (iii) Th-17 cytokines and their inducers, IL-17, transforming growth factor- β (TGF- β). We hypothesized that FS in ELBW infants would be associated with decreased Th-1 cytokines, IL-17 or TGF- β , and/or elevated Th-2 cytokines in the first 3 wk of life as compared with infants with BS or NS.

RESULTS

Of 1,066 eligible infants, 89 developed FS, 368 developed BS without FS, and 609 did not have any episode of culture-positive sepsis (Figure 1). DBSs were available for 684 infants on day (d)0, 847 infants on d3, 940 infants on d7, 892 infants on d14, and 735 infants on d21. Sepsis was diagnosed on basis of positive blood culture in 91 to 92%, CSF culture in 5 to 6%, and both blood and CSF culture in 2 to 3% of infants in both the BS and FS groups. In the FS group, 61 infants also had positive bacterial cultures (68.5%) either before ($n = 32$) or after ($n = 29$) after the first positive fungal blood and/or CSF culture.

The most commonly identified bacteria included coagulase-negative staphylococci ($n = 189$), *Staphylococcus aureus* ($n = 34$), *Streptococci* ($n = 35$; Group B *Streptococcus* = 4), *Escherichia coli* ($n = 20$), and *Klebsiella* ($n = 17$). Identified fungal elements included *Candida albicans* ($n = 44$), *Candida parapsilosis* ($n = 33$), *Candida* (species not specified, $n = 9$), *Candida glabrata* ($n = 2$), and *Saccharomyces* ($n = 1$).

Maternal and Neonatal Characteristics

Median (interquartile range) age at first sepsis episode was 18 (11–30.5) d in the BS group and 20 (14–32) in the FS group. In 55% of infants, sepsis was diagnosed between d7 and d21 of age. Maternal characteristics that differed significantly across sepsis group included preterm labor, hypertension, use of intrapartum antibiotics, and mode of delivery (Table 1). As compared with infants with NS, infants with BS or FS were more likely to have lower birthweight and gestational age, require delivery room intubation, require treatment with postnatal steroids, have three or more central lines, undergo assisted ventilation

and treatment with supplemental oxygen, have higher mortality, and have longer hospital stays in survivors. They were also more likely to develop necrotizing enterocolitis and patent ductus arteriosus and receive treatment with histamine-2 receptor (H_2) blockers.

Bivariate Analysis of Mean Cytokine Levels by Sepsis Group at Each Time Point

For each infant with BS or FS, DBSs were classified as being “preinfection” if obtained before the microbiologic confirmation of sepsis and as “postinfection” if obtained after microbiologic confirmation of sepsis (Table 2). Bivariate analysis of mean cytokine levels in the preinfection phase revealed significant group differences for IL-10 (d3, d14, d21); IL-18 (d14, d21); IFN- γ (d21), and TGF- β (d14) (Figures 2 and 3). In the postinfection phase, mean cytokine levels were significantly different between the three groups for IL-10 (d7, d14, d21), IL-18 (d14, d21), TGF- β (d14, d21), and TNF- α (d21). IL-10, IL-18, IFN- γ , and TNF- α levels were higher and TGF- β levels were lower in infants with FS as compared with infants with BS or NS (data for TNF- α not presented in Figure 2).

Mixed-Model Analysis: Interaction of Sepsis Group and Cytokines Over Time

On mixed-model analysis with adjustment for confounding variables, significant group differences, and postnatal time trends were observed for IFN- γ , IL-10, IL-18, TGF- β , and TNF- α , with significant interaction between group and age at cytokine evaluation (Table 3). There were significant differences in IFN- γ , IL-10, IL-18, and TGF- β between the pre- and postinfection periods, with a significant interaction for sepsis group. Table 4 summarizes the cytokines that were significantly different between sepsis groups in the pre- and postinfection periods on adjusted analyses.

Receiver Operating Characteristic Analysis

Receiver operating characteristic analysis revealed that three cytokines—IL-10, IL-18, and TGF- β —had significant ability to discriminate between sepsis groups in the pre- and postinfection phases on d14 and d21 (Table 5). We did not attempt to determine cutoff values for these cytokines at different time points, because in this study DBSs were obtained at predefined postnatal ages and not in relation to timing of onset of clinical suspicion of infection or positive blood culture. We computed area under the curve, which showed that all three cytokines had moderate to high accuracy (area under the curve ≥ 0.7) for differentiating between FS vs. NS and FS vs. BS in the postinfection phase on d14 and d21 DBS samples (Figure 4). Results in the preinfection phase for these cytokines were less robust.

DISCUSSION

To our knowledge, this is the first population study evaluating cytokine profiles in ELBW infants with NS, BS, or FS. Although cytokine levels and acute-phase reactants have been used as markers of neonatal sepsis, the response of these biomarkers to FS has not been systematically studied (14). In this

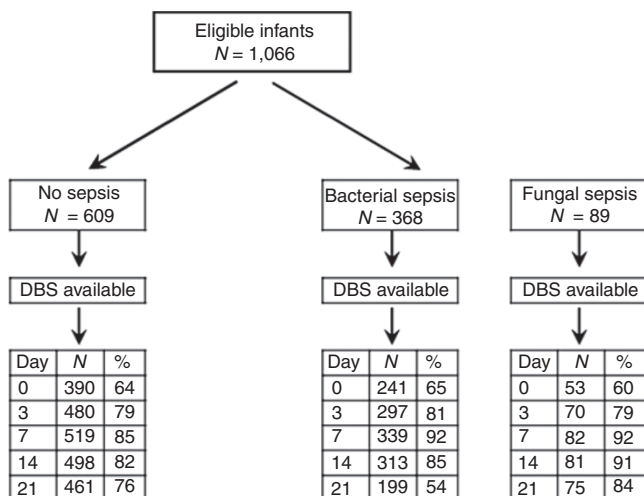


Figure 1. Flow diagram of preterm neonates with available DBSs by sepsis group. DBS, dried blood spot.

Table 1. Maternal and neonatal characteristics

Characteristic	Sepsis			<i>p</i> ^a	<i>p</i> ^b
	None N = 609	Bacterial N = 368	Fungal N = 89		
Preterm labor (%)	65	72	81	0.0013	0.0939
Hypertension (%)	28	23	7	<0.0001	0.0004
Maternal antibiotics before delivery (%)	66	71	81	0.0087	0.0603
Mode of delivery—cesarean section (%)	61	58	44	0.0067	0.0147
Gestational age (wk) (mean ± SD)	26 ± 2	26 ± 2	25 ± 2	<.0001	0.0001
Birth weight (gm) (mean ± SD)	777 ± 138	749 ± 141	699 ± 138	<.0001	0.0022
Five-minute Apgar ≤5 (%)	6	11	7	0.0329	NS
Delivery room intubation (%)	73	77	87	0.0143	0.0465
Postnatal steroids (%)	20	31	39	<0.0001	NS
Ventilator days (mean ± SD)	20 ± 23	33 ± 2	46 ± 29	<.0001	<0.0001
Days in oxygen (mean ± SD)	48 ± 38	64 ± 39	74 ± 34	<.0001	0.0306
Early sepsis (%)	0	4	3	<0.0001	NS
Late sepsis (%)	0	96	97	<0.0001	NS
Three or more central lines (%)	44	63	83	<0.0001	0.002
Grade III or IV IVH ^c (%)	15	18	24	0.0991	NS
BPD (%)	42	56	63	<0.0001	NS
NEC (≥stage 2) (%)	8	15	10	0.0025	NS
Deaths (%)	16.2	19.6	29.2	0.0100	0.0480
Age at discharge for survivors (d) (mean ± SD)	90 ± 32	113 ± 49	129 ± 54	<0.0001	0.0241
Patent ductus arteriosus (%)	42	57	75	<0.0001	0.0014
H2 blockers (%)	33	47	49	<0.0001	NS
Age at first positive culture (d) (mean ± SD)		25 ± 20	20 ± 16		0.033

Characteristics that did not show significant differences across sepsis groups included premature prolonged rupture of membranes, use of antenatal corticosteroids or tocolytics, gender, race, and cystic PVL.

BPD, bronchopulmonary dysplasia; H2, histamine-2 receptor; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis NS, not significant; PVL, periventricular leukomalacia.

^aComparison of all three sepsis groups. ^bBacterial vs. fungal sepsis.

Table 2. Number of infants with DBSs obtained before and after first episode of bacterial and fungal sepsis

Infection phase	Bacterial sepsis					Fungal sepsis				
	d0	d3	d7	d14	d21	d0	d3	d7	d14	d21
Preinfection	240	283	320	209	136	52	69	78	65	40
Postinfection	1	14	19	104	163	1	1	4	16	35
Total	241	297	339	313	199	53	70	82	81	75

d, day; DBS, dried blood spot.

exploratory, hypothesis-generating study, we identified a panel of five inflammatory markers significantly associated with sepsis group in the first 3 wk of life on adjusted analysis—IFN- γ , IL-10, IL-18, TGF- β , and TNF- α . These cytokines are potential candidate biomarkers of neonatal BS and FS that may allow early diagnosis before blood cultures become positive and that may also differentiate between BS and FS. Cytokines were not significantly different between groups on d0, suggesting that fetal inflammatory response syndrome is neither a marker for nor predisposes to neonatal sepsis and that systematic difference in cytokine profiles do not exist between infants who later acquire sepsis (BS or FS) and those who do not. Significant

group differences in cytokines in the preinfection period suggest that changes in levels of these cytokines may precede clinical signs and symptoms and microbiologic confirmation of diagnosis. After the microbiologic diagnosis of sepsis, group differences in cytokine levels became more prominent as compared with values before microbiologic confirmation, suggesting that these differences reflect a response to infection rather than conferring an inherent predisposition to infection.

In keeping with our hypothesis, IL-10 (Th-2 cytokine) was increased and TGF- β decreased in infants with FS as compared with those with BS or NS. However, contrary to our hypothesis, Th-1 cytokines, IFN- γ , IL-18, and TNF- α , were increased

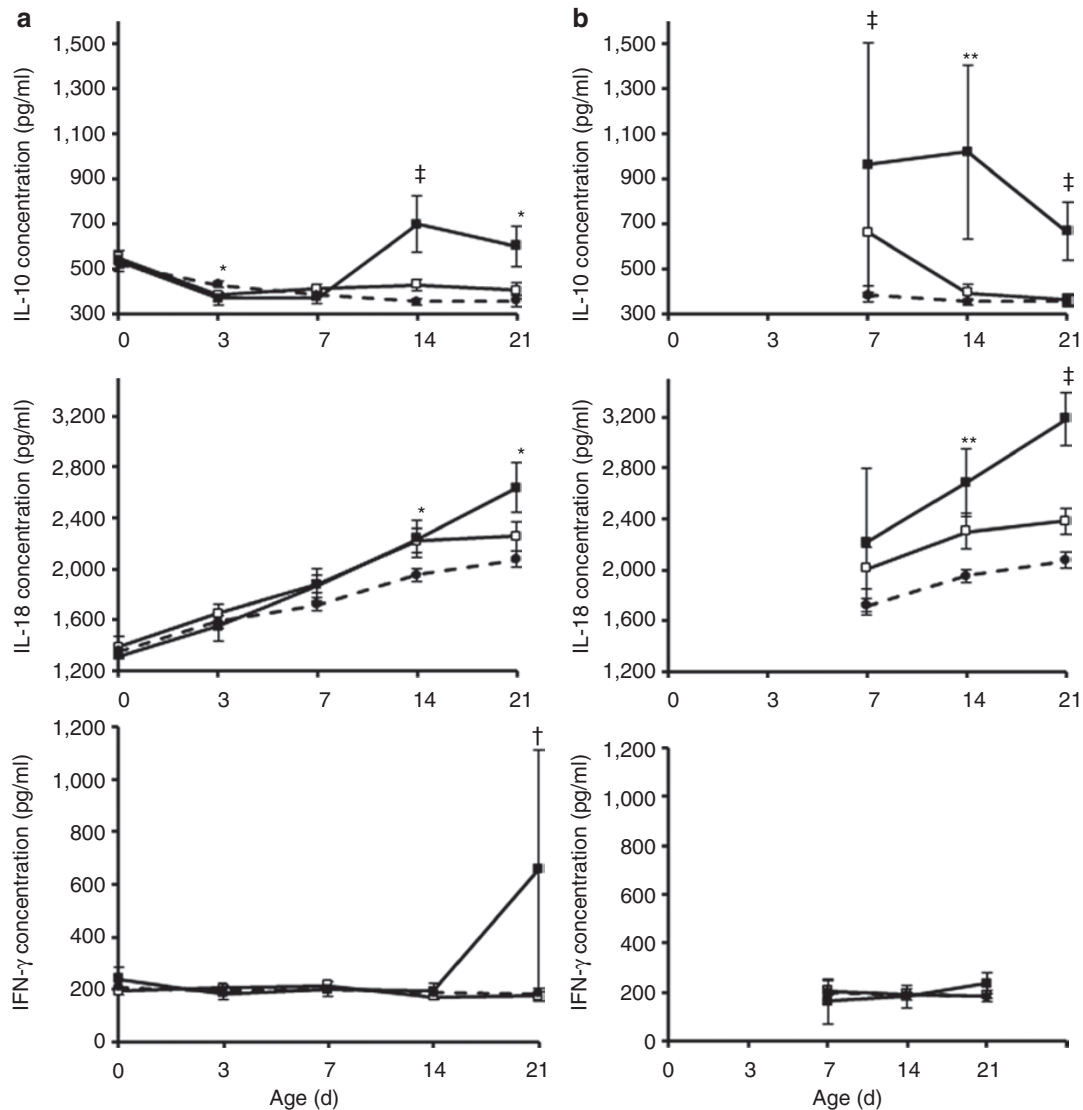


Figure 2. Mean concentrations (pg/ml) of cytokines significantly higher in fungal sepsis group before and after first episode of infection. (a) Data for pre-infection phase; (b) data for postinfection phase. Data for d0 and d3 not presented for postinfection period as there were very few cases. Refer to [Table 2](#) for number of cases in each group at various time points. Vertical lines represent ± 1 SE. * $P < 0.05$, ** $P < 0.01$, † $P < 0.001$, ‡ $P < 0.0001$. White squares, bacterial group; black squares, fungal group; black circles, no sepsis group. D, day; IL, interleukin; IFN- γ , interferon- γ .

in infants with FS. IL-1 β , IL-12, IL-4, IL-5, IL-6, and IL-17 did not show any association with sepsis group in our study. Our hypotheses were based on cytokine profiles described in sepsis in experimental models and adults. Only one observational study of FS and BS in a small group of term and preterm neonates has shown significant differences in C-reactive protein and IL-6 levels (15). Our results support the complexity of the immune response involving multiple cytokines with divergent functions and suggest that the immune response may differ in ELBW neonates.

TNF- α , initiator of the inflammatory response, has been reported to be increased early in neonatal sepsis. These studies included small numbers of patients, did not differentiate between FS and BS (16–18), were not limited to ELBW infants (19,20), and included infants with culture-negative sepsis (21,22). In our study TNF- α was significantly higher in ELBW infants with FS as compared with BS and NS.

IL-6, one of the most studied cytokines in neonatal sepsis, increases early, but its half-life is short and its sensitivity decreases after 12 to 24 h of infection. Most studies included small numbers of patients, did not differentiate between FS and BS (16,18,23–25), were not limited to ELBW infants (19,20,26–32), and included infants with culture-negative sepsis (33). In one study, IL-6 levels were significantly elevated in BS as compared with FS in a cohort of term and preterm infants (15). The lack of association of IL-6 with culture-proven BS and FS in our large homogeneous population of ELBW infants suggests that IL-6 may not play a role in immune response to sepsis in ELBW infants. Alternatively, imprecision in timing of collection of samples in relation to clinical suspicion of infection in our study may have led to spuriously negative results because of the short half-life of IL-6.

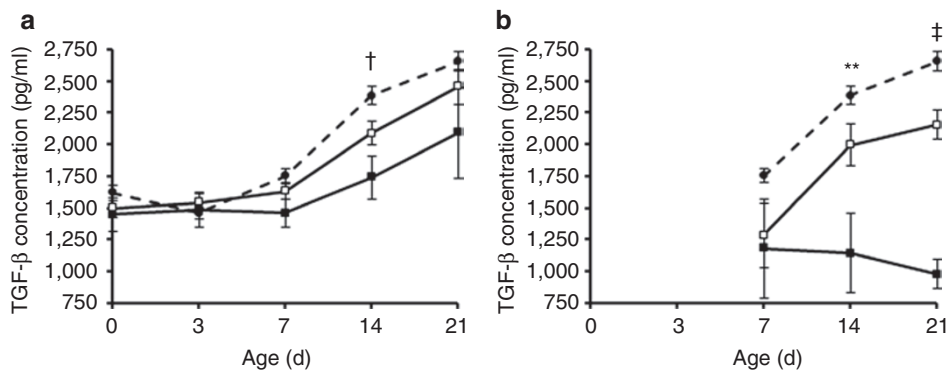


Figure 3. Mean concentrations (pg/ml) of cytokines significantly lower in fungal sepsis group before and after first episode of infection. (a) Data for pre-infection phase; (b) data for postinfection phases. Data for d0 and d3 not presented for postinfection period because there were very few cases. Refer to **Table 2** for number of cases in each group at various time points. Vertical lines represent ± 1 SE. ** $P < 0.01$, † $P < 0.001$, ‡ $P < 0.0001$. White squares, bacterial group; black squares, fungal group; black circles, no sepsis group. d, day; TGF- β , transforming growth factor- β .

Table 3. Mixed-model analysis for cytokines showing significant sepsis group and time interaction after controlling for significant covariates

Cytokine	Group	Effect – F value (probability > F)			Pre- vs. postinfection status	Pre-/postinfection status* group ^b	Significant covariates (P)
		Postnatal age	Group*age ^a				
IFN- γ	0.011	<0.0001	<0.0001	0.0005	0.0001	Center [†] Preterm labor*	
IL-10	0.0002	<0.0001	<0.0001	0.0075	0.0067	Center [†] Oxygen** NEC*	
IL-18	0.0097	<0.0001	0.0283	0.0009	0.0265	Center [†] GA** Hypertension**	
TGF- β	0.0024	<0.001	0.0007	<0.0001	0.0013	Center [†] GA** Preterm labor** PDA [†] NEC**	
TNF- α	0.0162	0.0025	0.0064	NS	NS	Center [†] PDA*	

GA, gestational age; IFN- γ , interferon- γ ; IL, interleukin; NS, not significant; PDA, patent ductus arteriosus; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α .

^aInteraction of group and age. ^bInteraction of pre- or postinfection status and group. * $P < 0.05$. ** $P < 0.01$. † $P < 0.001$. ‡ $P < 0.0001$.

IL-17, the signature cytokine of TH-17 cells, has an important role in the induction of neutrophil-mediated protective immune response against extracellular bacterial or fungal pathogens such as *Klebsiella pneumoniae* and *Candida albicans* in experimental models (11,34,35). However, in ELBW subjects of our study, IL-17 levels were not significantly different across sepsis group.

IL-18 is a proinflammatory cytokine that reduces polymorphonuclear cell apoptosis, potentiates IFN- γ production, and induces production of TNF- α , IL-1 β , and IL-8 (36). IL-18 is protective against infection caused by a number of intracellular and extracellular pathogens. In a recent comprehensive study of >140 serum analytes from neonates evaluated for late-onset sepsis (including BS, FS, and culture-negative clinical sepsis), IL-18 was elevated in infected vs. noninfected neonates and in FS (37). In our study as well, IL-18 emerged as a potential biomarker to differentiate FS from NS and BS.

IL-10 is an anti-inflammatory cytokine responsible for downregulation of the inflammatory process and maintaining homeostasis in vital organs (36). Elevated levels of IL-10 indicate infectious disease in neonates, and IL-10 remains elevated until 24 h after the beginning of sepsis (5,26,38). In our study, IL-10 discriminated not only between BS and NS but also between FS and BS or NS.

No previous studies have investigated levels of TGF- β , a bipolar cytokine that can both trigger and inhibit the immune system, in neonatal sepsis (39). In our study, ELBW infants with sepsis had lower levels of TGF- β on d14 as compared with infants with NS in the preinfection period. In the postinfection period, TGF- β levels were significantly different on d14–d21 between FS and BS or NS and between BS and NS.

On receiver operating characteristic analysis, three cytokines demonstrated moderate to high accuracy in discriminating between FS and NS or BS after microbiologic confirmation

of diagnosis despite the fact that DBSs were obtained at pre-defined postnatal ages and not timed in relation to first positive blood culture. These robust trends suggest that these cytokines may be biomarkers of neonatal FS and BS and need to be validated in prospective studies with blood samples obtained in relation to time of clinical suspicion of infection and first positive blood culture.

The majority of clinical characteristics that were strongly associated with sepsis group in our study have been previously reported (1). Center difference was a significant covariate in most of the analyses. This could be related to differing ethnic

populations, proportions of inborn/outborn infant, clinical practices, or other unidentified factors. Wide variation in the incidence of invasive fungal infections between neonatal intensive care units has been shown in multiple publications (3,40). Of note, even after controlling for these covariates, the association between FS and five cytokines remained significant, suggesting their role in the pathogenesis of FS. Postnatal time trends and gestational age differences for cytokines have been described by our group previously (41).

This is the first population based study to compare cytokine concentrations in the first 3 wk of life in ELBW infants with FS, BS or NS. Two small studies have previously explored the cytokine signature of host response to different microbiologic agents in neonates with elevated IL-18 and C-reactive protein and lower IL-6 levels as being hallmarks of fungal infections (15,37). Strengths of our study include a large sample size; prospectively collected data permitting a thorough investigation of the association of sepsis group and cytokine response at pre-defined time points; standardized collection of repeated, timed blood samples for 3 wk starting from birth; assay of multiple cytokines from small volumes of blood using the Luminex assay; and multivariate adjustment for control of clinical variables.

Despite the important findings of this study, there are limitations. Although infants with BS represented a pure group with no positive fungal blood/CSF cultures, 68.5% of infants with FS also had positive blood/CSF bacterial cultures before or after the first episode of FS. Positive blood cultures for bacteria during the period of fungal infection are characteristic of the natural history of disease of invasive fungal infections in ELBW infants (1). Contamination of FS group with BS should have underestimated differences between the two

Table 4. Summary of cytokines that show statistically significant differences by age and sepsis group on adjusted analyses

	Before first episode of sepsis				After first episode of sepsis		
	d3	d7	d14	d21	d7	d14	d21
BS vs. NS			IL-10 IL-18 TGF-β		IL-10	IL-10 IL-18 TGF-β	IL-18 TGF-β
FS vs. NS	TGF-β		IL-10 IL-18 TGF-β	IFN-γ IL-10 IL-18 TNF-α	IL-10 TGF-β	IL-10 IL-18 TGF-β TNF-α	IL-10 IL-18 TGF-β TNF-α
FS vs. BS			IL-10	IFN-γ IL-10 TNF-α		IL-10 IL-18 TGF-β TNF-α	IL-10 IL-18 TGF-β TNF-α

In analysis before first episode of infection, no group differences in cytokines on D0 observed. In analysis after first episode of infection, group differences were not evaluated at d0 and d3 because of the small numbers of subjects at these time points. All cytokines except TGF-β were elevated in BS or FS vs. NS and in FS vs. BS. TGF-β was decreased in BS or FS vs. NS and in FS vs. BS.

BS, bacterial sepsis; d, day; FS, fungal sepsis; IFN-γ, interferon-γ; IL, interleukin; NS, no sepsis; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

Table 5. Area under the curve for cytokines significantly different between groups in the pre- and postinfection phases

Cytokine	Analysis	d14			d21		
		BS vs. NS	FS vs. NS	BS vs. FS	BS vs. NS	FS vs. NS	BS vs. FS
Preinfection phase							
IL-10	Unadjusted	0.56 (0.51–0.60)**	0.59 (0.51–0.67)**	0.54 (0.45–0.62)**			0.61 (0.51–0.72)*
	Adjusted	0.72 (0.69–0.77)†	0.81 (0.76–0.86)‡			0.80 (0.74–0.86)*	0.70 (0.61–0.79)*
IL-18	Unadjusted	0.56 (0.52–0.61)*				0.68 (0.59–0.76)*	
	Adjusted	0.61 (0.56–0.65)*					
TGF-β	Unadjusted	0.56 (0.51–0.60)*	0.64 (0.57–0.71)**			0.67 (0.58–0.77)*	
	Adjusted						
Postinfection phase							
IL-10	Unadjusted		0.73 (0.59–0.87)**	0.71 (0.56–0.86)*			0.58 (0.45–0.69)**
	Adjusted		0.87 (0.76–0.98)**	0.79 (0.65–0.92)*		0.85 (0.78–0.91)*	0.81 (0.73–0.89)**
IL-18	Unadjusted	0.58 (0.51–0.64)*	0.72 (0.62–0.82)*		0.59 (0.54–0.64)*	0.78 (0.72–0.85)‡	0.70 (0.61–0.79)**
	Adjusted	0.66 (0.61–0.72)*			0.61 (0.56–0.66)*	0.85 (0.79–0.91)**	0.80 (0.73–0.87)*
TGF-β	Unadjusted	0.60 (0.53–0.66)*	0.79 (0.66–0.92)**		0.60†	0.85‡	0.78‡
	Adjusted		0.91 (0.82–1.0)*		0.63*	0.92‡	0.85†

Numbers represent AUC (95% confidence limits).

AUC, area under the curve; BS, bacterial sepsis; FS, fungal sepsis; IL, interleukin; NS, no sepsis; TGF-β, transforming growth factor-β.

*P < 0.05. **P < 0.01. †P < 0.001. ‡P < 0.0001.

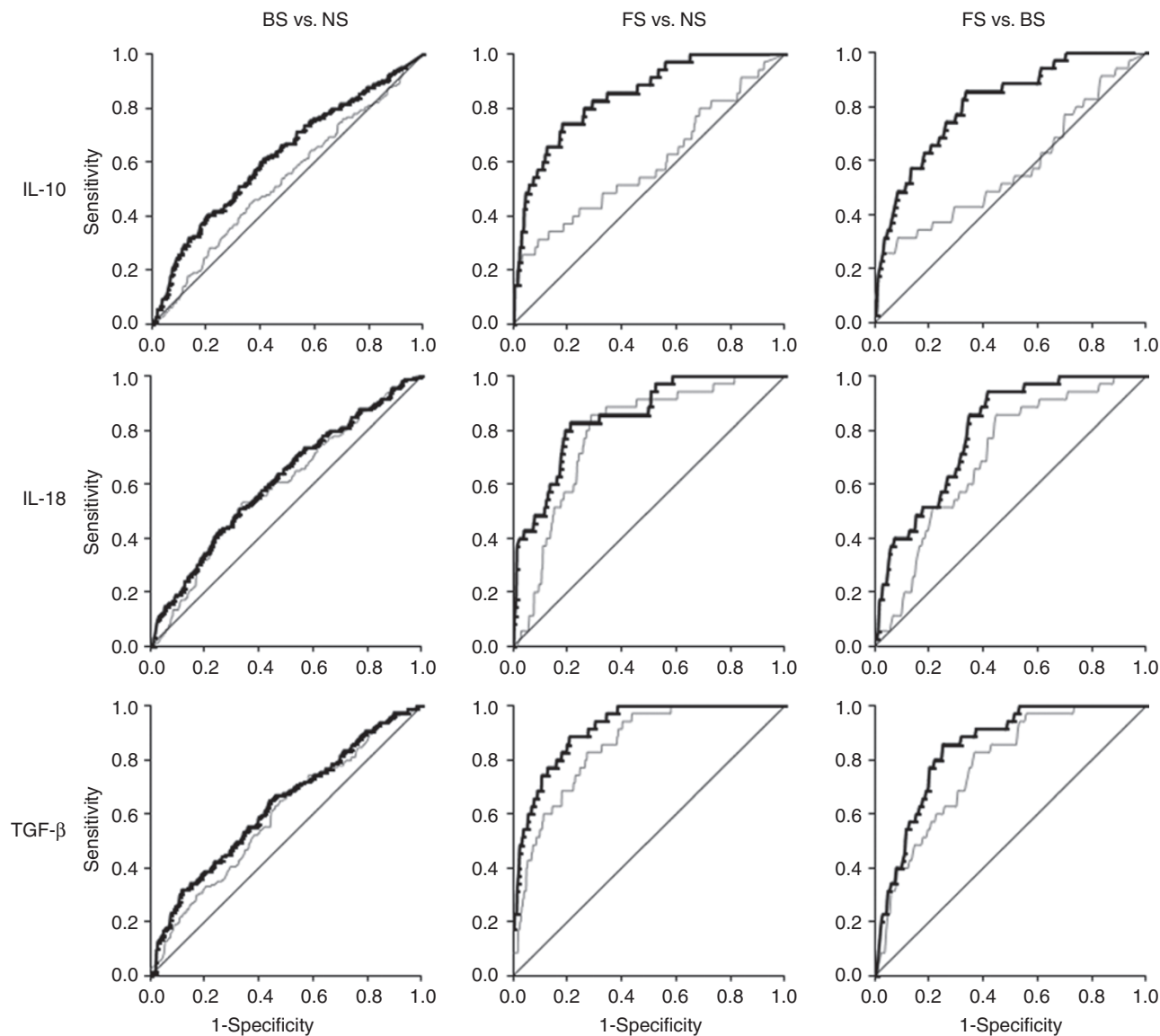


Figure 4. ROC curves for cytokines significantly different between sepsis groups in the postinfection period on d21. Gray and bold black lines represent ROC curve in analyses unadjusted and adjusted for significant covariates, respectively. BS, bacterial sepsis; d, day; FS, fungal sepsis; IL, interleukin; NS, no sepsis; ROC, receiver operating characteristic; TGF- β , transforming growth factor- β .

groups; in spite of that we were able to detect significant differences between groups for five cytokines, suggesting that these reflect true differences in cytokine response in BS and FS. Second, blood samples were timed in relation to postnatal age and not to clinical suspicion of infection. It has been shown that cytokine levels decline rapidly after onset of infection. Therefore DBSs obtained at specified postnatal ages may have occurred at different time intervals before or after onset of infection. Despite this imprecision, we obtained consistent and significant predictive values for three cytokines at 14–21 d of age, which is close to the median age of onset of confirmed sepsis, suggesting dramatic effects. Although the study design of collection of protocol-driven samples at predefined postnatal ages in this hypothesis-generating secondary study was not ideal to assess response to infection, it did allow evaluation of cytokine levels before diagnosis of

sepsis, which may not be possible to assess in a study design in which sample collection is dictated by clinical suspicion of infection. Although these results may reflect true differences between FS, BS, and NS because of the robust trends and biologic plausibility, they need to be validated in carefully designed large longitudinal cohort studies with prospective blood collection in relation to sepsis evaluation. Third, it is difficult to distinguish between primary and secondary mediators in the cytokines described. In addition, circulating cytokine concentrations may not reflect their biologic activity. Although we included 11 biomarkers, other markers (procalcitonin, C-reactive protein) that have been shown to be useful for the early detection of neonatal sepsis were not studied (5,37). Last, we did not correct for multiple comparisons because the *post hoc* subgroup analysis in this study is exploratory in nature and hypothesis-generating. Therefore,

reliance should be placed on the observed effect size rather than on statistical significance testing as a basis for decision making (42).

In conclusion, in this prospective study, IL-10, IL-18, TGF- β , and TNF- α were found to be the most promising indicators of FS vs. BS or NS in ELBW infants. These findings offer insight into immune regulation in ELBW infants with sepsis, offering the prospect of rapid differentiation of neonatal BS and FS, early institution of targeted antimicrobial therapy, and development of new treatments for this disease by enhancing the production of cytokines that confer protection. These novel findings will require validation in larger rigorously designed prospective studies.

METHODS

We conducted a secondary exploratory analysis using clinical and biologic data collected as part of the multicenter National Institute of Child Health and Human Development Cytokine Study, a prospective study assessing biomarkers for adverse neurodevelopmental outcomes in preterm infants with a birthweight of 401–1,000 g (43). This study was approved by the institutional review boards at all 17 participating centers, and written informed consent was obtained from parent(s).

ELBW neonates who were enrolled in the National Institute of Child Health and Human Development Cytokine Study and had available DBSs were eligible for the study. Sepsis (including early and late-onset) was defined by a blood and/or CSF culture positive for bacteria or fungi and ≥ 5 d of treatment (or intent to treat in those who died before 5 d). The outcome variable was sepsis group—No (NS), bacterial (BS, ≥ 1 episode of bacterial sepsis without FS), and fungal (FS, ≥ 1 episode of FS). Infants with positive bacterial and fungal blood/CSF culture were included in the FS group as positive blood cultures for bacteria during the period of fungal infection are characteristic of the natural history of disease of invasive fungal infections in ELBW infants (1). Cultures positive for *Corynebacterium* spp., *Propionibacterium* spp., *Alcaligenes* spp., *Penicillium* spp., and diptheroids were considered contaminants and excluded. Cultures positive for coagulase-negative *Staphylococcus* were reviewed and categorized as a definite infection, possible infection, or probable contaminant as described previously (14). Definite and possible coagulase-negative *Staphylococcus* infections were included as sepsis whereas probable contaminants were not. Infants who had cultures positive only for pathogens considered contaminants were included in the noninfected group.

Whole blood was collected on filter paper and frozen at five time periods (d): 0–1 (d0), 3 \pm 1 (d3), 7 \pm 2 (d7), 14 \pm 3 (d14), and 21 \pm 3 (d21). The treatment of blood samples was standardized. After the filter paper blood sample had dried, it was wrapped in a stock paper cover and placed in a plastic bag, which was stored in a -20°C freezer as soon as possible after the specimen was dry, but no later than 24 h. Clinical information was collected by trained research nurses on all morbidities following preterm birth and their risk factors, permitting a thorough investigation of the association of inflammatory cytokines and sepsis group.

Cytokines were analyzed in stored blood spots using a multiplex Luminex assay (Luminex, Austin, TX) as described previously (44,45). This assay has low intra- (<10%) and inter-assay (7–23%) variation. It has been shown that cytokine measurements on DBSs stored at -24°C for >20 y are stable over time. For this secondary analysis of the primary study, 11 cytokines were identified as being important in the development of FS (TNF- α , IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IL-18, IFN- γ , and TGF- β).

For each infant with BS or FS, DBS, and cytokine levels were classified as preinfection if they were obtained before the first episode of bacterial sepsis in the BS group or before the first episode of FS in the FS group and postinfection if they were collected after the first episode of infection.

Statistical Analyses

Statistical analyses were performed using SAS System 9.2 (SAS, Cary, NC). Unadjusted analysis was performed using χ^2 tests for categorical outcomes and ANOVA test (*F*-test) for continuous outcomes to study the association of several maternal and neonatal variables with sepsis group (NS, BS, and FS). Medians and interquartile ranges were computed for all cytokines at each of the five time points and bivariate analyses used to assess differences across sepsis groups. For each cytokine, levels were plotted against time for each sepsis group to assess their temporal profiles over the first 3 postnatal wk. Differences in temporal profiles across sepsis groups were evaluated using mixed-effect models for each cytokine independently to account for the multicenter design, repeated measurement of cytokine levels, significant clinical covariates, and interaction of postnatal age and sepsis group. Confounding variables included center, gestational age, birthweight, race, gender, premature prolonged rupture of membranes, antenatal steroids, postnatal steroids, days in oxygen, intraventricular hemorrhage (grades 3, 4), cystic periventricular leukomalacia, surgically treated patent ductus arteriosus, and necrotizing enterocolitis (Bell stage ≥ 2). A *P* value of <0.05 was considered statistically significant. A receiver operating characteristic curve was constructed to determine the cytokines that could predict sepsis group. Area under the curve was determined to measure accuracy of various cytokines in discriminating between sepsis groups.

ACKNOWLEDGMENTS

The results reported in this manuscript were presented in part at the Pediatric Academic Societies' Annual Meeting on 2 May 2011 at Denver, CO, USA. We are indebted to our medical and nursing colleagues and the infants and their parents who agreed to take part in this study. Data collected at participating NRN sites were transmitted to RTI International, the data coordinating center (DCC) for the NRN, which stored, managed, and analyzed the data for this study. A.D. (DCC principal investigator) and S.S. (DCC statistician) had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis.

STATEMENT OF FINANCIAL SUPPORT

The National Institutes of Health (General Clinical Research Center grants M01 RR30, M01 RR32, M01 RR39, M01 RR70, M01 RR80, M01 RR633, M01 RR750, M01 RR997, M01 RR6022, M01 RR7122, M01 RR8084, and M01 RR16587), the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) (grants U10 HD36790, U10 HD21364, U10 HD21373, U10 HD21385, U10 HD21397, U10 HD21415, U10 HD27851, U10 HD27853, U10 HD27856, U10 HD27871, U10 HD27880, U10 HD27881, U10 HD27904, U10 HD34216, U10 HD40461, U10 HD40492, U10 HD40498, U10 HD40689), and the U.S. Centers for Disease Control and Prevention (Interagency Agreement Y1-HD-5000-01) provided grant support for the Neonatal Research Network's Cytokines Study. In addition, D.K.B. received support from the Thrasher Research Fund and NICHD (Grant HD44799). The funding agencies provided overall oversight for study conduct, but all data analyses and interpretation were independent of the funding agencies. Information regarding investigator/agency participation can be found in the **Supplementary Data** online.

REFERENCES

1. Kaufman D, Fairchild KD. Clinical microbiology of bacterial and fungal sepsis in very-low-birth-weight infants. *Clin Microbiol Rev* 2004;17:638–80, table of contents.
2. Kaufman DA, Cuff AL, Wamstad JB, et al. Fluconazole prophylaxis in extremely low birth weight infants and neurodevelopmental outcomes and quality of life at 8 to 10 years of age. *J Pediatr* 2011;158:759–765.e1.
3. Stoll BJ, Hansen N, Fanaroff AA, et al. Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics* 2002;110(2 Pt 1):285–91.
4. Stoll BJ, Hansen NI, Adams-Chapman I, et al.; National Institute of Child Health and Human Development Neonatal Research Network. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *JAMA* 2004;292:2357–65.

5. Zeitoun AA, Gad SS, Attia FM, Abu Maziad AS, Bell EF. Evaluation of neutrophilic CD64, interleukin 10 and procalcitonin as diagnostic markers of early- and late-onset neonatal sepsis. *Scand J Infect Dis* 2010;42:299–305.
6. Hohl TM, Rivera A, Pamer EG. Immunity to fungi. *Curr Opin Immunol* 2006;18:465–72.
7. Berger A. Th1 and Th2 responses: what are they? *BMJ* 2000;321:424.
8. Yu JJ, Gaffen SL. Interleukin-17: a novel inflammatory cytokine that bridges innate and adaptive immunity. *Front Biosci* 2008;13:170–7.
9. Ghilardi N, Ouyang W. Targeting the development and effector functions of TH17 cells. *Semin Immunol* 2007;19:383–93.
10. Huang W, Na L, Fidel PL, Schwarzenberger P. Requirement of interleukin-17A for systemic anti-*Candida albicans* host defense in mice. *J Infect Dis* 2004;190:624–31.
11. Acosta-Rodriguez EV, Rivino L, Geginat J, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 2007;8:639–46.
12. Shen F, Gaffen SL. Structure-function relationships in the IL-17 receptor: implications for signal transduction and therapy. *Cytokine* 2008;41:92–104.
13. Pappu BP, Angkasekwinai P, Dong C. Regulatory mechanisms of helper T cell differentiation: new lessons learned from interleukin 17 family cytokines. *Pharmacol Ther* 2008;117:374–84.
14. Schelonka RL, Maheshwari A, Carlo WA, et al.; NICHD Neonatal Research Network. T cell cytokines and the risk of blood stream infection in extremely low birth weight infants. *Cytokine* 2011;53:249–55.
15. Oguz SS, Sipahi E, Dilmen U. C-reactive protein and interleukin-6 responses for differentiating fungal and bacterial aetiology in late-onset neonatal sepsis. *Mycoses* 2011;54:212–6.
16. Caldas JP, Marba ST, Blotta MH, Calil R, Morais SS, Oliveira RT. Accuracy of white blood cell count, C-reactive protein, interleukin-6 and tumor necrosis factor alpha for diagnosing late neonatal sepsis. *J Pediatr (Rio J)* 2008;84:536–42.
17. Ucar B, Yildiz B, Aksit MA, et al. Serum amyloid A, procalcitonin, tumor necrosis factor-alpha, and interleukin-1beta levels in neonatal late-onset sepsis. *Mediators Inflamm* 2008;2008:737141.
18. Ng PC, Li K, Leung TF, et al. Early prediction of sepsis-induced disseminated intravascular coagulation with interleukin-10, interleukin-6, and RANTES in preterm infants. *Clin Chem* 2006;52:1181–9.
19. Hotoura E, Giapros V, Kostoula A, Spirou P, Andronikou S. Tracking changes of lymphocyte subsets and pre-inflammatory mediators in full-term neonates with suspected or documented infection. *Scand J Immunol* 2011;73:250–5.
20. Kurt AN, Aygun AD, Godekmerdan A, Kurt A, Dogan Y, Yilmaz E. Serum IL-1beta, IL-6, IL-8, and TNF-alpha levels in early diagnosis and management of neonatal sepsis. *Mediators Inflamm* 2007;2007:31397.
21. Shouman B, Badr R. Regulated on activation, normal T cell expressed and secreted and tumor necrosis factor-alpha in septic neonates. *J Perinatol* 2010;30:192–6.
22. Ng PC, Li K, Chui KM, et al. IP-10 is an early diagnostic marker for identification of late-onset bacterial infection in preterm infants. *Pediatr Res* 2007;61:93–8.
23. Küster H, Weiss M, Willeitner AE, et al. Interleukin-1 receptor antagonist and interleukin-6 for early diagnosis of neonatal sepsis 2 days before clinical manifestation. *Lancet* 1998;352:1271–7.
24. Gonzalez BE, Mercado CK, Johnson L, Brodsky NL, Bhandari V. Early markers of late-onset sepsis in premature neonates: clinical, hematological and cytokine profile. *J Perinat Med* 2003;31:60–8.
25. Ng PC, Li K, Wong RP, et al. Proinflammatory and anti-inflammatory cytokine responses in preterm infants with systemic infections. *Arch Dis Child Fetal Neonatal Ed* 2003;88:F209–13.
26. Cancelier AC, Petronilho F, Reinke A, et al. Inflammatory and oxidative parameters in cord blood as diagnostic of early-onset neonatal sepsis: a case-control study. *Pediatr Crit Care Med* 2009;10:467–71.
27. Onal EE, Kitapçi F, Dilmen U, Adam B. Interleukin-6 concentrations in neonatal sepsis. *Lancet* 1999;353:239–40.
28. Hodge G, Hodge S, Haslam R, et al. Rapid simultaneous measurement of multiple cytokines using 100 microl sample volumes—association with neonatal sepsis. *Clin Exp Immunol* 2004;137:402–7.
29. Messer J, Eyer D, Donato L, Gallati H, Matis J, Simeoni U. Evaluation of interleukin-6 and soluble receptors of tumor necrosis factor for early diagnosis of neonatal infection. *J Pediatr* 1996;129:574–80.
30. Romagnoli C, Frezza S, Cingolani A, et al. Plasma levels of interleukin-6 and interleukin-10 in preterm neonates evaluated for sepsis. *Eur J Pediatr* 2001;160:345–50.
31. Panero A, Pacifico L, Rossi N, Mancuso G, Stegagno M, Chiesa C. Interleukin 6 in neonates with early and late onset infection. *Pediatr Infect Dis J* 1997;16:370–5.
32. Döllner H, Vatten L, Austgulen R. Early diagnostic markers for neonatal sepsis: comparing C-reactive protein, interleukin-6, soluble tumour necrosis factor receptors and soluble adhesion molecules. *J Clin Epidemiol* 2001;54:1251–7.
33. Laborada G, Rego M, Jain A, et al. Diagnostic value of cytokines and C-reactive protein in the first 24 hours of neonatal sepsis. *Am J Perinatol* 2003;20:491–501.
34. Matsuzaki G, Umemura M. Interleukin-17 as an effector molecule of innate and acquired immunity against infections. *Microbiol Immunol* 2007;51:1139–47.
35. Conti HR, Shen F, Nayyar N, et al. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med* 2009;206:299–311.
36. Oppenheim JJ, Ruscetti FW. Cytokines. In: Parslow TG, Stites DP, Terr AI, Imboden JB, eds. *Medical Immunology*, 10th edn. New York: Lange Medical Books/McGraw-Hill, 2001:148–166.
37. Kingsmore SE, Kennedy N, Halliday HL, et al. Identification of diagnostic biomarkers for infection in premature neonates. *Mol Cell Proteomics* 2008;7:1863–75.
38. Bender L, Thaarup J, Varming K, Krarup H, Ellermann-Eriksen S, Ebbesen F. Early and late markers for the detection of early-onset neonatal sepsis. *Dan Med Bull* 2008;55:219–23.
39. Wahl SM. Transforming growth factor-beta: innately bipolar. *Curr Opin Immunol* 2007;19:55–62.
40. Benjamin DK Jr, Stoll BJ, Gantz MG, et al.; Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network. Neonatal candidiasis: epidemiology, risk factors, and clinical judgment. *Pediatrics* 2010;126:e865–73.
41. Sood BG, Madan A, Saha S, et al.; NICHD neonatal research network. Perinatal systemic inflammatory response syndrome and retinopathy of prematurity. *Pediatr Res* 2010;67:394–400.
42. Lagakos SW. The challenge of subgroup analyses—reporting without distorting. *N Engl J Med* 2006;354:1667–9.
43. Carlo WA, McDonald SA, Tyson JE, et al.; Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network. Cytokines and neurodevelopmental outcomes in extremely low birth weight infants. *J Pediatr* 2011;159:919–25.e3.
44. Skogstrand K, Ekelund CK, Thorsen P, et al. Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples. *J Immunol Methods* 2008;336:78–84.
45. Skogstrand K, Thorsen P, Nørgaard-Pedersen B, Schendel DE, Sørensen LC, Hougaard DM. Simultaneous measurement of 25 inflammatory markers and neurotrophins in neonatal dried blood spots by immunoassay with xMAP technology. *Clin Chem* 2005;51:1854–66.