

Expression of Serum Cytokines Profile in Neonatal Sepsis

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Abstract

Sepsis remained a major cause of neonatal death, but the pathologic mechanisms were poorly understood. The objective of this study was to characterize the serum cytokine/chemokine profile in neonates with sepsis. In this study, we enrolled 40 full-term neonates with sepsis and 19 neonates without infection as controls. Serum 40 cytokines/chemokines were analyzed using Luminex Bead Immunoassay System. Serum IL-17 was measured using enzyme-linked immune-absorbent assay. Our results showed that serum IL-6, IL-8, TNF- α , IL-1 β , MIF, CXCL13, CXCL1, CXCL2, CXCL5, CXCL6, CXCL16, CCL27, CCL2, CCL8, CCL3, CCL20, CCL23, CCL27 and CX3CL1 were significantly increased in neonates with sepsis compared to controls (all $p < 0.05$). The levels of serum CXCL6, CXCL1, IL-6, CXCL10, CXCL11, CCL20, and IL-17 were higher in LOS than those in EOS (all $p < 0.05$). Conversely, serum IL-16, CXCL16, CCL22 were lower in LOS than those in EOS (all $p < 0.05$). The levels of CX3CL1, CXCL2, CCL8 and TNF- α were all positively correlated with SOFA scores. We suggest that excessive pro-inflammatory cytokines might involve in the damage of neonatal sepsis. In addition, chemokines significantly increased the recruitment of immune cells after infection to participate in the anti-infection defense of neonates, but they might lead to damage.

Introduction

Neonatal sepsis is a systemic infection of bacterium, virus, or fungus characterized by life-threatening organ dysfunction [1]. Despite advances in the management of neonates and the new generation of antibiotics, sepsis is still the leading cause of neonatal deaths, with more than 1 million deaths worldwide each year [2–4].

After infection, pathogen-associated molecular patterns (PAMPs) are recognized by sentinel immune cells through several classes of pathogen recognition receptors (PRRs) (e.g. Toll-like receptors) [5–7]. Activation of these receptors can stimulate the release of inflammatory mediators, including cytokines and chemokines. Therefore, the clinical features of neonatal sepsis were present as systemic inflammatory response syndrome (SIRS). Several studies have demonstrated that serum pro-inflammatory TNF- α , IL-1 β , IL-6, and IL-8 were rapidly and strikingly elevated in neonatal sepsis [8–10]. The levels of serum CXCR4 and CXCL12 in neonatal sepsis were significantly higher than those in controls [11, 12]. The level of CXCL10 was increased in the blood and peritoneum in a murine model of neonatal polymicrobial sepsis [13]. Furthermore, these cytokines could be used as valuable biomarkers for the diagnosis of neonatal sepsis [8–12]. However, studies on the relationship between cytokine/chemokine levels and severity, and the difference of cytokines/chemokines between early-onset sepsis (EOS) and late-onset sepsis (LOS) are limited. Moreover, little work has been done to assess the dramatic changes in innate immunity and adapt immunity during neonatal sepsis. Therefore, in order to better characterize the inflammatory response during neonatal sepsis, we systematically analyzed cytokine/chemokine profiles in neonatal sepsis in the present study.

Material And Methods

Ethics statement and subjects

The study was approved by the Ethical Committee of the Second Affiliated Hospital of Wenzhou Medical University. Between October 2016 and June 2018, eligible neonates who were hospitalized for sepsis at the neonatal intensive care unit (NICU) of the Second Affiliated Hospital of Wenzhou Medical University were asked to participate in this study. Written informed consent was obtained from their parents or legal guardians. 40 full-term neonates with sepsis were enrolled in this study and 19 neonates without clinical manifestations or maternal risk factors for infection were as the control group. Neonates with congenital malformations, having received antibiotics or undergoing surgery were excluded.

The diagnostic criteria of neonatal sepsis

Confirmed neonatal sepsis was defined as a positive blood culture accompanied by compatible signs and symptoms. Suspected neonatal sepsis was defined as the presence of laboratory findings suggestive of infection (neutrophilia/neutropenia, thrombocytopenia, elevated CRP, and ESR) in combination with at least three of the following symptoms and signs without other causes: temperature instability (core temperature ≥ 38.5 or $\leq 36^\circ\text{C}$); respiratory symptoms (apnoea, tachypnoea with respiratory rate > 60 per minute, cyanosis, need for high ventilator settings or oxygen); cardiovascular symptoms including hypotension (blood pressure < 5 th percentile for age), tachycardia (heart rate > 160 per minute), bradycardia (heart rate < 80 per minute), or poor perfusion; neurological symptoms (hypotonia, hyporeflexia, irritability, lethargy and seizures); gastrointestinal symptoms (poor feeding, abdominal distension, green or bloody residuals, vomiting). Early-onset sepsis (EOS) was onset in the first 72 hours after birth, late-onset sepsis (LOS) was onset more than or equal to 72 hours after birth. Organ dysfunction and severity were scored according to Sequential Organ Failure Assessment (SOFA).

Quantification of serum cytokines and chemokines

3 ml of venous blood from all participants was collected and centrifuged at 3000rpm for 15 minutes. Serum was stored at -70°C until analysis. 40 cytokines and chemokines including CCL21, CXCL13, CCL27, CXCL5, CCL11, CCL24, CCL26, CX3CL1, CXCL6, GM-CSF, CXCL1, CXCL2, CCL1, IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, CXCL10, CXCL11, CCL2, CCL8, CCL7, CCL13, CCL22, MIF, CXCL9, CCL3, CCL15, CCL20, CCL19, CCL23, CXCL16, CXCL12, CCL17, CCL25, TNF- α were analyzed using the Luminex Bead Immunoassay System (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Serum IL-17 levels were measured using human IL-17 Quantikine ELISA kits (eBioscience) according to the manufacturer's instruction.

Statistical analysis

Statistical analyses were performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA). Categorical variables were expressed as frequency and percentage. Continuous variables were presented as median (interquartile range, IQR). Statistical significance in serum cytokine and chemokine levels between different groups was assessed by the Mann-Whitney U test. Differences between before and after

treatment were analyzed by using the paired *t* test. For correlation analysis, Spearman's correlation coefficients were calculated. Statistical significance in this study was set at $p < 0.05$ and all reported *p*-values were 2-sided.

Results

1. The clinical characteristics of the enrolled neonates

Fifty-nine full-term neonates were enrolled in this study, including 40 neonates with sepsis (30 EOS and 10 LOS) and 19 uninfected neonates as controls. Temperature was 38.1°C (IQR 37.13°C-38.65°C) and 36.8°C (IQR 36.5°C-37.2°C) in neonates with sepsis and control, respectively ($p < 0.0001$). In 13 neonates with positive blood culture, the following pathogens were isolated: *Escherichia coli* (7 cases), *Streptococcus lactis* (4 cases), *Klebsiella* (1 case) and *Enterococcus faecium* (1 case). The levels of serum C reactive protein (CRP) and Serum amyloid protein A (SAA) in neonates with sepsis reached 9.11 mg/ml (IQR 6.54 mg/ml-10.13 mg/ml) and 29.38 mg/ml (IQR 4.83 mg/ml-36.24 mg/ml), respectively, which were significantly higher compared to the control group (both $p < 0.0001$). The neutrophil counts of sepsis and control neonates were $9.06 \times 10^9/L$ (IQR $5.34 \times 10^9/L$ - $14.84 \times 10^9/L$) and $6.14 \times 10^9/L$ (IQR $4.29 \times 10^9/L$ - $7.76 \times 10^9/L$) ($p = 0.018$), respectively. There was no significant difference in the counts of monocytes, lymphocytes, and platelets between the two groups (all $p > 0.05$). The detailed information of the clinical characteristics was shown in Table 1.

Table 1
The clinical characteristics of the enrolled neonates

Variable	Neonate sepsis (n = 40)	Control (n = 19)	p value
Age (Median, IQR), days	12(3.25–19.75)	10(7-16.25)	0.672
Male gender, n (%)	25(62.5)	13(68.4)	0.775
Temperature (°C)	38.1(37.13–38.65)	36.8(36.5–37.2)	< 0.0001
Blood culture, n (%)	13(32.5)	nd	nd
Early of sepsis, n (%)	30(75)	0	nd
WBC($\times 10^9/L$)	17(11.68–21.13)	12.1(9.9–14.7)	0.005
Neutrophils($\times 10^9/L$)	9.06(5.34–14.84)	6.14(4.29–7.76)	0.018
Monocytes($\times 10^9/L$)	1.49(0.90–2.05)	1.26(0.96–51.46)	0.189
lymphocytes($\times 10^9/L$)	4.07(3.14–5.53)	4.22(3.43–5.02)	0.942
Platelets($\times 10^9/L$)	316.5(249–436)	262(227–345)	0.076
CRP (mg/ml)	9.11(6.54–10.13)	0.35(0.18–0.69)	< 0.0001
SAA (mg/ml)	29.38(4.83–36.24)	0.38(0.04–3.86)	< 0.0001
Note: nd, not done; WBC, White Blood Cell; CRP, C reactive protein; SAA, serum amyloid protein A. Values are given as median (interquartile range).			

2. The levels of serum cytokines and chemokines in neonatal sepsis

We compared serum cytokines and chemokines levels between neonates with sepsis and controls. Since the level of CCL15 was not in the quantitative range of the assay and exceeded the highest calculated concentration, we did not analyze it further. As shown in Table 2, pro-inflammatory cytokines IL-6, IL-8, TNF- α , and IL-1 β were significantly higher in neonates with sepsis than those in controls (all $p < 0.05$). There was no difference in the levels of IL-4, IFN- γ , IL-17, IL-2, IL-10, GM-CSF, and IL-16 between the two groups (all $p > 0.05$). Serum MIF was remarkably increased in neonates with sepsis, which was 6270 pg/ml (IQR 4615 pg/ml-11820 pg/ml) and 4947 pg/ml (IQR 3204 pg/ml-6767 pg/ml) in sepsis and controls, respectively ($p = 0.0223$). As shown in Table 3, serum CXCL13, CXCL1, CXCL2, CXCL5, CXCL6, CXCL16, CCL27, CCL2, CCL8, CCL3, CCL20, CCL23, CCL27, CX3CL1 were significantly increased in neonates with sepsis compared to controls (all $p < 0.05$).

Table 2

The serum levels of cytokines in control group and neonatal sepsis group.

Cytokine	Control group	Sepsis group	p value
GM-CSF (pg/ml)	125.2(87.8-234.3)	149.4(96.62–228.9)	0.4533
IFN- γ (pg/ml)	105.5(78.75–167.2)	107.8(76.35–160.6)	0.6977
IL-1β (pg/ml)	10.93(5.955–11.22)	12.02(10.68–18.19)	0.0071
IL-2 (pg/ml)	30.52(14.01–46.2)	36.81(24.28–45.02)	0.2794
IL-4 (pg/ml)	38.02(25.45–42.63)	36.1(30.99–43.43)	0.418
IL-6 (pg/ml)	24.25(12.9-39.01)	58.57(37.03–236.6)	< 0.0001
IL-8 (pg/ml)	38.5(17.45–64.08)	262.1(130.1–630)	< 0.0001
IL-10 (pg/ml)	89.27(42.78–134.7)	95.2(67.34–137.3)	0.2871
IL-16 (pg/ml)	1176(617.8–1832)	1005(727.2–1331)	0.3417
MIF (pg/ml)	4947(3204–6767)	6270(4615–11820)	0.0223
TNF-α (pg/ml)	45.72(26.27–55.92)	53.14(44.26–79.38)	0.0213
IL-17 (pg/ml)	1.13(0-2.525)	1.792(0.3479–5.423)	0.1723

Table 3

The chemokine levels of serum in control group and neonatal sepsis group.

Chemokine	Control group (n = 19)	Sepsis group (n = 40)	p value
CCL21(pg/ml)	7380(4948–15970)	6356(3958–9688)	0.1434
CXCL13(pg/ml)	53.38(41.35–72.76)	86.9(51.3-142.8)	0.003
CCL27(pg/ml)	1565(945–2621)	2041(1385–2895)	0.0361
CXCL5(pg/ml)	969.7(550.4–1366)	1187(929.4–1947)	0.0321
CCL11(pg/ml)	63.4(50.19–89.31)	77(58.22–89.35)	0.4947
CCL24(pg/ml)	263(122.4-485.8)	231.1(124.8-356.8)	0.4493
CCL26(pg/ml)	70.31(46.26–102.6)	77.38(58.56–98.76)	0.3283
CX3CL1(pg/ml)	365.9(223.1-584.3)	609.7(529.9-857.1)	< 0.0001
CXCL6(pg/ml)	48.4(30.76–95.48)	88.18(54.28–108.4)	0.005
CXCL1(pg/ml)	277.9(244.3-332.5)	436.8(359.2–763)	< 0.0001
CXCL2(pg/ml)	143.3(54.57–315.1)	331.3(186.3-700.9)	0.0027
CCL1(pg/ml)	112.6(96.16–145.3)	116.7(88.1-153.7)	0.7378
CXCL10(pg/ml)	171.4(87.76–262.7)	210(97.66–593.5)	0.185
CXCL11(pg/ml)	23.95(7.658–36.5)	20.28(13.38–53.64)	0.8251
CCL2(pg/ml)	77.22(28.18–166.8)	168(89.07–363.4)	0.0011
CCL8(pg/ml)	32.67(21.28–39.7)	61.6(40.28–101.8)	< 0.0001
CCL7(pg/ml)	204(145.8-377.9)	238.5(151.5-370.6)	0.5557
CCL13(pg/ml)	53.52(12.8-87.72)	47.06(22.71–91.9)	0.7478
CCL22(pg/ml)	1694(1061–3421)	1234(774.3–1615)	0.0829
CXCL9(pg/ml)	658.3(251.5-960.4)	604.7(394.3–1069)	0.4413
CCL3(pg/ml)	16.28(9.845–30.3)	58.16(32.58–136.5)	< 0.0001
CCL20(pg/ml)	25.24(13.16–42.4)	50.93(20.63–97.86)	0.0074
CCL19(pg/ml)	902.2(274.9–1714)	772.5(466.5–1381)	0.8356
CCL23(pg/ml)	273.6(107.9-609.3)	1092(554.5–2366)	< 0.0001
CXCL16(pg/ml)	965.4(774.3–1152)	1218(1105–1364)	0.0004
CXCL12(pg/ml)	1559(423–1790)	1215(410.4–1607)	0.279

Chemokine	Control group (n = 19)	Sepsis group (n = 40)	p value
CCL17(pg/ml)	269.7(100.5-999.8)	417.5(122.4-1024)	0.705
CCL25(pg/ml)	1156(698.6-1498)	1285(895.3-1792)	0.3123

Subsequently, we further analyzed the differences of these cytokines and chemokines between EOS and LOS. As shown in Table 4, the levels of serum CXCL6, CXCL1, IL-6, CXCL10, CXCL11, CCL20, and IL-17 were higher in LOS than those in EOS (all $p < 0.05$). Conversely, the levels of serum IL-16, CXCL16, CCL22 were lower in LOS than those in EOS (all $p < 0.05$).

Table 4
The levels of serum cytokines and chemokines between EOS and LOS.

Cytokine/ Chemokine	EOS (n = 10)	LOS (n = 30)	p value
CXCL6(pg/ml)	66.55(37.7-97.69)	93.27(61.68-121.5)	0.0299
CXCL1(pg/ml)	370.1(266-449.8)	528.6(393.9-957.2)	0.0408
IL-6(pg/ml)	44.18(32.08-57.81)	87.68(44.9-354.5)	0.0479
IL-16(pg/ml)	1449(1099-1948)	885(607.4-1211)	0.0015
CXCL10(pg/ml)	110.9(62.27-268.6)	253.3(136.2-705.1)	0.0415
CXCL11(pg/ml)	11.81(5.853-20.38)	22.48(16.25-62.85)	0.0152
CCL22(pg/ml)	1485(1179-3912)	1164(619.3-1440)	0.0276
CCL20(pg/ml)	22.86(14.3-40.77)	62.76(32.03-124.9)	0.0093
CXCL16(pg/ml)	1403(1169-1532)	1207(1007-1334)	0.0351
IL-17(pg/ml)	0.43(0-0.97)	2.89(1.16-6.47)	0.003

There were 13 neonates with positive blood culture and 27 neonates with negative blood culture among 40 neonates with sepsis. We compared the levels of serum cytokines and chemokines between positive blood culture and negative blood culture. The levels of CXCL6, TNF- α , CCL8, CCL23 in neonates with positive blood culture were significantly higher than those with negative blood culture (all $p < 0.05$) (Table 5). There was no difference in the levels of other cytokines and chemokines between the two groups (date not shown).

Table 5
The chemokine and cytokine levels of serum in blood culture positive group and blood culture negative group.

Cytokine/ Chemokine	Positive (n = 13)	Negative (n = 27)	p value
CXCL6(pg/ml)	99.88(65.67–157.9)	82.05(51.1-102.2)	0.0444
CCL8 (pg/ml)	92.06(49.62–375.7)	43.81(38.66–76.02)	0.0373
CCL23(pg/ml)	1954(528–3292)	746.2(366.1–1223)	0.0311

3. The levels of cytokines and chemokines were decreased after treatment

The serum of 15 neonates with sepsis before and after treatment were collected. 41 cytokines and chemokines were measured. As shown in **Supplemental Digital Content – Fig. 1**, IL-6, IL-8, TNF- α , IL-1 β , CXCL13, CXCL16, CCL27, CCL3, CCL23, CX3CL1 were significantly decreased after treatment (all $p < 0.005$).

4. The association between the levels of cytokines and chemokines and the severity of organ function.

We further analyzed the associated between the levels of cytokines and chemokines and the SOFA score. As shown in **the Supplemental Digital Content – Fig. 2**, the levels of CX3CL1, CXCL2, CCL8, and TNF- α were all positively correlated with SOFA scores (all $p < 0.05$).

Discussion

Neonatal sepsis is the major risk factor for neonatal mortality [14]. Furthermore, the pathological mechanism remains unclear. In the present study, we systematically investigated the dynamic changes of cytokine and chemokine profiles in term neonatal sepsis. In accordance with previous studies [8–10], pro-inflammatory cytokines IL-6, IL-8, TNF- α , and IL-1 β were significantly increased in neonates with sepsis. A moderate increase in cytokines plays a protective role and promotes antimicrobial immune responses, whereas excessive upregulation of pro-inflammatory cytokines is commonly associated with a severe and often fatal outcome due to multiple organ failure [15]. Our results showed that the level of TNF- α was positively correlated with SOFA scores, suggesting that TNF- α might involve in the organ damage in neonates with sepsis.

Chemokine is a family of cytokines and has the capacity to recruit leukocytes to pathogen invasion sites, which is essential for the host to defend against infection [16]. Our results showed that serum CXCL13, CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, CXCL16, CCL27, CCL2, CCL8, CCL3, CCL20, CCL23, CCL27 and CX3CL1 were significantly increased in neonates with sepsis than controls. *Manoura A et al* also found that the levels of serum CXCL1 and CXCL5 were higher in neonates with sepsis [17]. Among these chemokines, CXCL1, CXCL2, CXCL5, CXCL6, and CXCL8 are potent chemo-attractants for neutrophils. Neutrophils are the most abundant cells of the innate immunity and play an important role in the response and defense against bacterial, viral, and fungal infections [18]. Previous studies had shown that

elastase and nitric oxide in neonates with sepsis were upregulated [19, 20]. In our study, the counts of neutrophils in neonates with sepsis were higher than those in controls. These results suggested that neutrophils-related chemokines would be immediately synthesized and released after infection, which might subsequently lead to recruited neutrophils to participate in the defense of neonates against infection.

The defense of neonates is initially dependent on the innate immune, because adaptive immunity will develop in later life [21]. Interestingly, in our study, Th17 cell-related cytokine/chemokine IL-17 and CCL20, and Th1 cell-related chemokines CXCL10 and CXCL11 were significantly higher in LOS compared to those of EOS. Our results indicated that pro-inflammatory cytokines were initially released in neonates at the onset of infection, but the adaptive immunity had gradually developed in the late stage of infection.

The serum MIF of neonatal sepsis was significantly higher than that of the control group. After treatment, MIF decreased significantly. The MIF of newborns was 10-fold higher than that of children and adults. *E. coli* and *Group B Streptococcus* induced MIF secretion by newborn monocytes [22]. MIF played an important role in promoting the production of inflammatory cytokines by monocytes [23]. Previous studies indicated that MIF was correlated with the expression of proinflammatory markers, dysregulated pituitary and adrenal function, severity scores, and disease outcomes [24–27]. Therefore, MIF might play critical roles in the immune regulation of neonatal sepsis.

In our study, serum CX3CL1 was increased in neonates with sepsis. The level of CX3CL1 was positively correlated with SOFA scores. A previous study had reported that the level of plasma CX3CL1 was elevated in a mouse model of CLP-induced sepsis [28]. The level of CX3CL1 in adult sepsis patients increased with the severity and the number of organ dysfunctions [29]. Non-survivors had sustained elevated CX3CL1 levels compared to survivors [29]. Taken together, these data suggested that CX3CL1 was a risk factor for sepsis outcome. CX3CL1 acts through the CX3CR1 receptor and is a unique member of the CX3C chemokine family. CX3CR1 mainly expressed on CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ monocytes, which were represent an activated, more mature 'macrophage-like' subset and were greatly expanded in various infectious and inflammatory diseases and might be more than 50% in sepsis [30]. These data suggested that CX3CL1 might recruit activated monocytes and is involved in neonatal sepsis damage. However, the roles of CX3CL1 in neonatal sepsis need further study.

There are some limitations of this study. Firstly, the subjects enrolled are relatively small, and our findings need to be proven in future studies with a larger cohort. Secondly, gestational age is a essential factor for these cytokines and chemokines, therefore, we only enrolled full-term neonates in our study.

In conclusion, our study suggested that excessive inflammatory in neonatal sepsis might involve in the damage of neonatal sepsis. Neutrophils, monocytes, and lymphocytes associated chemokines increased significantly after infection. On the one hand, the recruited immune cells participated in the anti-infective defense of the neonates, on the other hand, it might cause damage.

Abbreviations

PAMP, pathogen-associated molecular patterns; SIRS, systemic inflammatory response syndrome; EOS, early-onset sepsis; LOS, late-onset sepsis; NICU, neonatal intensive care unit; TLR, Toll-like receptors; SOFA, Sequential Organ Failure Assessment;

Declarations

Conflicts of interest: No potential conflicts of interest were disclosed.

Conflicts of interest statement

Neither this paper nor any similar paper has been or will be submitted to or published in any other scientific journal. All authors are aware and agree with the content of the paper and agree to be listed as the author of the manuscript. There is no conflict of interest or competing financial interests for all authors.

Data Availability

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

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Figures

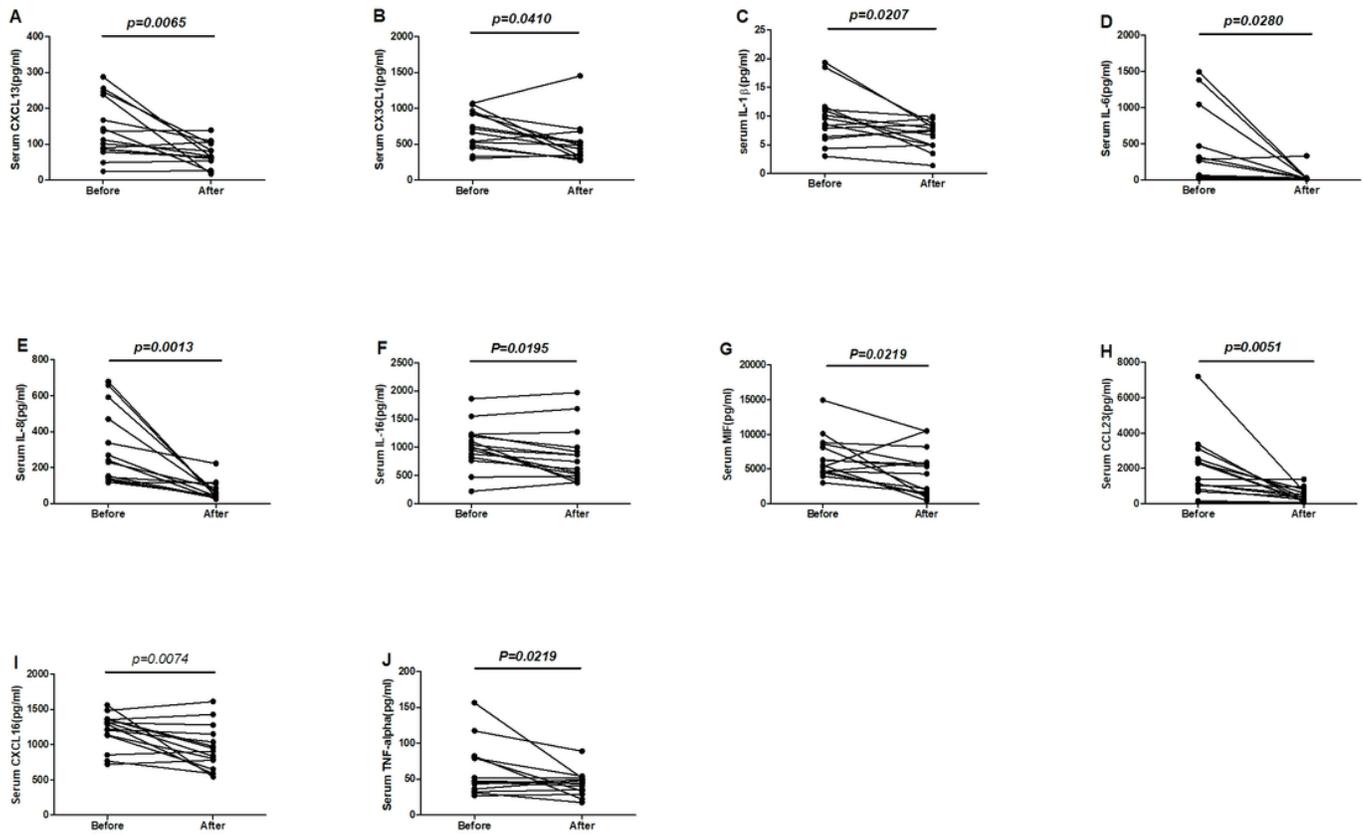


Figure 1

The levels of cytokines and chemokines before and after treatment in 15 neonates with sepsis.

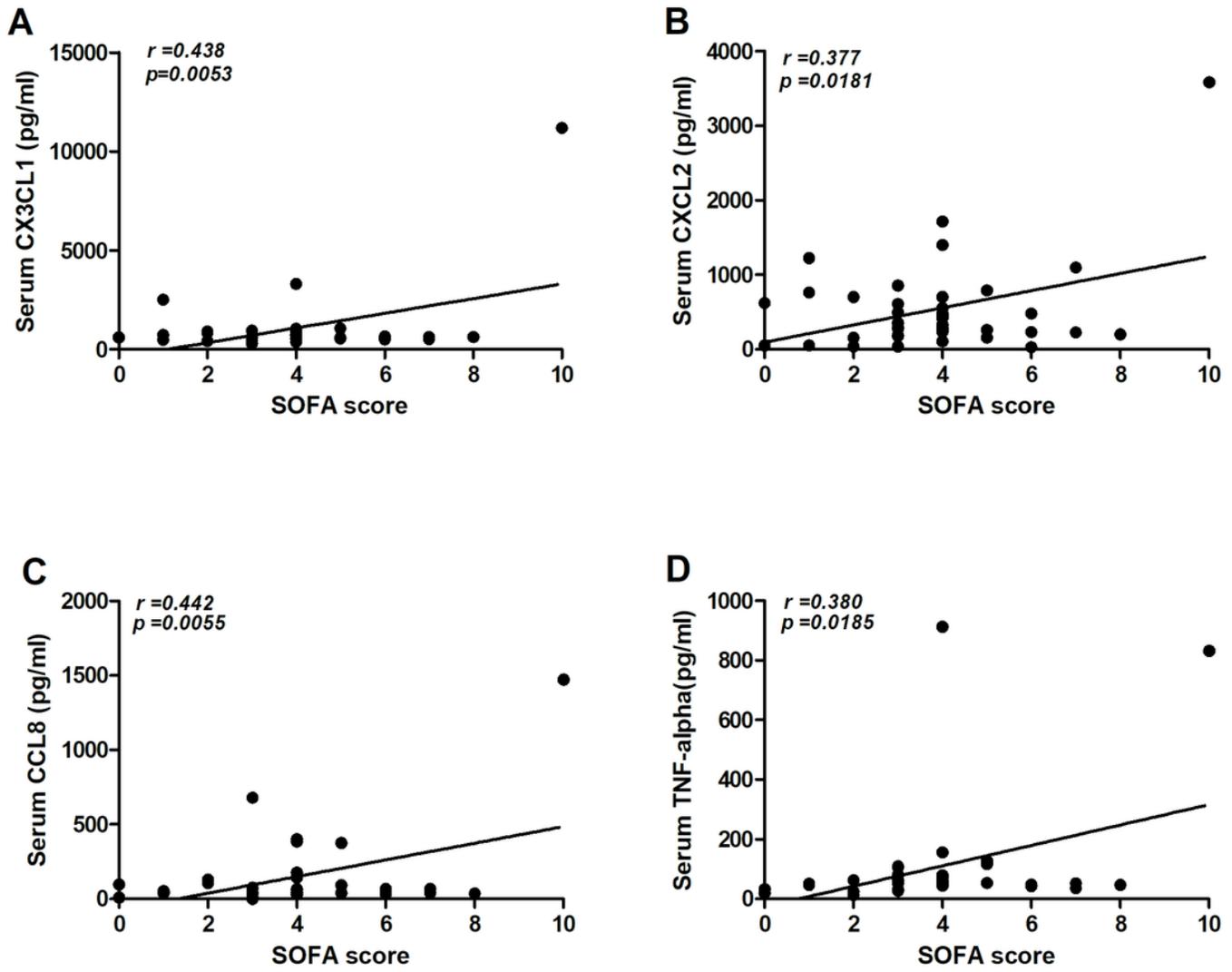


Figure 2

The association between the levels of cytokines and chemokines and SOFA score.