Early Human Development 129 (2019) 65–70

Contents lists available at ScienceDirect

Early Human Development

journal homepage: www.elsevier.com/locate/earlhumdev

Relationship between cytokine profiles of cord blood and cord S100B levels in preterm infants

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1. Introduction

Brain damage in the perinatal period has been implicated in the pathogenesis of both neurodevelopmental impairments and psychiatric illnesses. The profound vulnerability of the developing brain to cumulative insults by inflammatory, hypoxic-ischemic, and metabolic processes is the primary cause of fetal brain damage in prematurity [1]. Moreover, preterm infants exposed to inflammatory cytokines under conditions, such as chorioamnionitis (CAM), had an increased risk of brain injury [2,3]. Knowledge of the factors that contribute to fetal brain damage and constitute a major role in its pathogenesis would be crucial in perinatal care to prevent abnormal development. However, investigating the pathogenesis is challenging because fetal brain damages occur less frequently [4]. Thus, in the present study, we investigated the association of cord blood cytokine profiles and clinical factors with fetal brain damage by measuring cord blood S100B as a surrogate endpoint.

S100B is an acidic calcium-binding protein belonging to the EF-hand family concentrated in the nervous system, where it is located mainly in the glial cells [5]. Although a number of potential early markers of brain damage have been investigated in the last decades, the assay of the S100B in different biological fluids has proven to be the most reliable [6]. There are many reports on the correlation between the severity of neuronal injury and the concentration of S100B, and those investigating the utility of cord blood S100B. High concentrations of cord S100B have been reported in cases with perinatal asphyxia, hypoxic-ischemic encephalopathy (HIE) [7], and CAM [8]. However, to the best of our knowledge, the relationship between cord blood cytokines and fetal brain damage with respect to arterial cord blood S100B has not been previously investigated. The effects of other inflammatory mediators, such as chemokines and growth factors, on cord blood S100B are less well known. Therefore, we focused on determining the association between profiles of multiple cytokines present in cord blood and cord S100B concentrations in preterm infants.

2. Methods

2.1. Case registration and clinical data

From preterm deliveries recorded at our institution, we excluded the cases which had maternal or fetal complications, such as preeclampsia/eclampsia, gestational diabetes mellitus, major congenital anomalies, intrauterine fetal death, multiple pregnancies or other medical disorders. As a result, between January 2012 and December 2017, 151 eligible cases were selected. Moreover, the cases whose arterial cord blood could not be collected were not enrolled. Thus, the retrospective analysis was performed using data of 64 enrolled participants.

Perinatal and neonatal clinical data were obtained from the hospital records. Infertility treatment was defined to include artificial insemination and *in vitro* fertilization. Tocolysis was defined as the use of intravenous magnesium sulfate, ritodrine hydrochloride, or vaginal progesterone. Labor onset was defined as the onset of regular painful uterine contractions with cervical changes. Induction of labor was defined as the stimulation of uterine contractions before the labor onset (with or without rupture of fetal membranes). Non-reassuring fetal status (NRFS) was considered to be present if the fetal heart-rate pattern was at levels 3–5, based on the guidelines of the Japan Society of Obstetrics and Gynecology [9]. Gestational age-specific birth weight/height z-score in SD units was calculated using the LMS method [10] in each case with Japanese gestational age-specific reference for birth weight [11]. Histological CAM was defined as an infiltration of polymorphonuclear leukocytes in the chorioamniotic membrane.

2.2. Cord blood samples and the data

Arterial cord blood samples were collected immediately after delivery. After performing the gas analysis at the laboratory in our institution, the serum of the cord blood was promptly separated by centrifugation and stored in aliquots at −80 °C until analysis. The Human

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https://doi.org/10.1016/j.earlhumdev.2019.01.013
Received 8 November 2018; Received in revised form 7 January 2019; Accepted 16 January 2019

Cytokine Standard 17-Plex Assay (Bio-Rad Laboratories, Inc., Corp., Hercules, USA) was used to investigate the following cytokines (lower limits of detection for each cytokine): the *granulocyte-colony stimulating factor* (G-CSF; 1.50 pg/mL); granulocyte monocyte-colony stimulating factor (GM-CSF; 0.04 pg/mL); interferon-gamma (IFN-γ; 0.26 pg/mL); Interleukins (IL)-1β (0.07 pg/mL), IL-2 (0.20 pg/mL), IL-4 (0.02 pg/mL), IL-5 (0.83 pg/mL), IL-6 (0.11 pg/mL), IL-7 (0.14 pg/mL), IL-8 (1.03 pg/mL), IL-10 (0.38 pg/mL), IL-12 (0.22 pg/mL), IL-13 (0.05 pg/mL), and IL-17 (0.19 pg/mL); monocyte chemotactic protein-1 (MCP-1; 0.51 pg/mL); macrophage inflammatory protein 1-β (MIP-1β; 0.45 pg/mL); and tumor necrosis factor-α (TNF-α; 3.34 pg/mL) on the Luminex200 system (Luminex, USA) according to the manufacturer's instructions. A commercially available enzyme-linked immune sorbent assay (ELISA) kit (Wako Pure Chemical, Osaka, Japan) was used to determine the levels of S100B in the cord blood.

2.3. Statistical analysis

We conducted statistical analyses using SPSS version 25.0 for Windows (SPSS, Inc., Chicago, IL). Possible associations of the S100B concentrations with various characteristics and cord blood biomarkers were studied using univariate (Fisher's exact test or Mann-Whitney *U* test) and multivariate (logistic regression) analyses. Variables that showed statistical significance in the univariate analyses were considered in the multivariate regression models. The cord blood biomarkers, IL-2, IL-7, IL-12, IL-13, IL-17, and GM-CSF showed values below the lower measurable limits in at least one case, and thus they were transcribed for use as qualitative data. The data above the lower-limit values were considered positive and the remaining data negative. In the logistic regression analysis, the data of cord blood pH, base excess (BE), and S100B were divided into two groups, according to the average values previously reported (7.27, −2.7, and 2.09 ng/mL, respectively) [8]. We included the case with 2.09 ng/mL or more of S100B concentrations in the High S100B group, and the remaining cases were in the Low S100B group. In simple linear regression analysis, the values of cord blood pH, BE, and S100B were used as quantitative data. A p-value of < 0.05 was considered to be significant.

3. Results

Table 1 shows the obstetric findings of eligible, enrolled, and not enrolled cases. Although the rate of NRFS might be considered a little high in enrolled cases, the other confounding factors had no clinically significant differences. Table 2 shows the perinatal and neonatal clinical features of the 64 enrolled cases. Since our institution is a regional tertiary unit, the maternal age was somewhat high in both groups. The percentage of tocolysis was quite high, due to focusing on preterm birth. For the same reasons, the overall rate of cesarean section (CS) was also high, which was more significant in low S100B groups ($p < 0.001$). The incidence of maternal fever and labor onset was significantly higher in the high S100B groups ($p < 0.001$ and $p < 0.001$, respectively). The other characteristics, including gestational weeks at birth and the rate of histological CAM showed no statistically significant differences between the two study groups.

The distribution of the 17 cytokines and the data of the gas analysis in the cord blood are listed in Table 3. As expected, cord pH and BE were significantly reduced in the high S100B group than those in the low S100B group ($p = 0.009$ and $p = 0.041$, respectively). Among the pro-inflammatory cytokines, only the IL-1β concentration was significantly increased in the high S100B group ($p < 0.001$). The blood levels of Th1 cytokines and growth factors were considerably low, and no significant difference was observed. With respect to the Th2 cytokines, IL-4 was significantly increased in the high S100B group ($p = 0.031$). Although no obvious differences were noted, a trend toward an increase in the IL-17 and MCP-1 levels was detected in the high S100B group.

Moreover, to evaluate the correlation between the S100B levels and the related factors in univariate analysis, we conducted a simple linear regression analysis. Although IL-4 was not correlated ($p = 0.546$, $r = 0.077$) (data not shown), the values of umbilical artery pH, BE, and IL-1β had significant associations with S100B levels (pH, $p = 0.015$, $r = -0.303$; BE, $p = 0.002$, $r = -0.383$; IL-1β, $p = 0.008$, $r = 0.328$) (Fig. 1).

Additionally, we performed a multivariate logistic regression analysis, and found that the cord IL-1β level was independently associated with high S100B levels in cord blood (OR 6.589; 95% CI 1.126–38.55; $p = 0.036$) (Table 4).

4. Discussion

Inflammation is associated with preterm delivery and adverse neonatal outcomes [12,13]. However, to the best of our knowledge, no study had simultaneously examined a wide range of inflammatory mediators and analyzed their relationship to fetal brain damage with respect to concentrations of cord S100B. Our findings suggested that elevated S100B concentrations were associated not only with certain maternal and delivery characteristics, but also with cord blood cytokines, especially IL-1β.

Although fetal brain damage remains a major clinical problem in the world, the mechanism has not been fully discovered. One of the reasons is the relatively low morbidity and the difficulty in collecting appropriate clinical material. Zaigham et al. reported that during a 12-year study period, only 34 neonates with HIE were identified in their registers, corresponding to an incidence of 0.09% (34/36,550 total births) [7]. Takahashi et al. revealed the relationship between cytokine levels in cord blood and perinatal or neonatal findings; however, they could not analyze the relationship to periventricular leukomalacia (PVL), since there was only one case with PVL out of 224 [4]. This was quite similar in our institution. Thus, we investigated the impact of cord blood cytokine profiles and clinical factors on fetal brain damage by measuring cord blood S100B as the surrogate endpoint. The surrogate endpoint has been defined as a biomarker intended to substitute for a clinical endpoint [14]. S100B is released mainly from glial cells in response to neuronal injury. Moreover, studies have shown that an increase in its concentration is related to the severity of the neuronal injury and the risk of developing permanent sequelae [15–17]. Thus, it is reasonable to consider S100B as surrogate endpoint of fetal brain damage. According to the previous report [18], cord S100B concentrations have significant negative correlation with gestational weeks, and the 90th percentile value of S100B is slightly above 2 ng/mL at 33 gestational weeks. The median birth weeks in our study were 33 weeks in both groups (Table 2). Thus, consequently, our definition of the High S100B (≧2.09 ng/mL) could be considered to extract extremely high S100B cases.

Interleukin-1β, IL-6, and TNF-α are the proinflammatory cytokines that have been studied frequently in term [19,20] and preterm [21,22] infants. Most of these studies reported elevated levels of these mediators in the newborns with evidence of perinatal brain damage than those in the controls. In our study, among the various cytokines, IL-1β levels had a significant positive correlation with S100B concentration (Fig. 1) and the strongest causal link to high S100B in cord blood (Table 4). Proinflammatory cytokines directly exert a deleterious effect on the developing brain; injection of IL-1β leads to neuronal death and delayed myelination in animal experiments [23]. Moreover, IL-1β is one of the agents that is associated with altered blood brain barrier (BBB) integrity [24], and the immaturity of BBB leads to an increase in serum S100B concentrations [8]. Therefore, it is reasonable to consider that the increase of IL-1β could contribute to elevation of S100B in cord blood. A recent report suggested that, in the amniotic fluid, IL-1β had the highest sensitivity to premature brain injury [17], which is in accordance with our findings. In contrast, though some tendency could be found in TNF-α, concentrations of cord IL-6 had no significant effect on

Table 1
Characteristics of the eligible patients enrolled and not enrolled.

	Eligible		
	Enrolled	Not enrolled	Total eligible
	(*n* = 64)	(*n* = 87)	(*n* = 151)
Maternal characteristics			
Maternal age (years)	34.0 (31.0–37.3)	35.0 (31.0–37.5)	35.0 (31.0–37.5)
Primiparity	33 (51.6)	51 (58.6)	84 (55.6)
Infertility treatment	17 (26.6)	19 (21.8)	36 (23.8)
Body mass index	20.3 (19.1–22.4)	20.1 (18.6–22.5)	20.2 (18.8–22.4)
Maternal fever (> 37.5 °C)	6 (9.4)	9 (10.3)	15 (9.9)
Tocolysis	43 (67.2)	49 (56.3)	92 (60.9)
Magnesium sulfate	13 (20.3)	15 (17.2)	28 (18.5)
Delivery characteristics			
Gestational weeks at birth (weeks)	33.0 (30.0–35.0)	35.0 (32.0–36.0)	34.0 (31.0–36.0)
Labor onset	27 (42.2)	41 (47.1)	68 (45.0)
Preterm rapture of membrane	27 (42.2)	34 (39.1)	61 (40.4)
Fetal tachycardia (> 160 bpm)	4 (6.3)	6 (6.9)	10 (6.6)
Non-reassuring fetal status	16 (25.0)	7 (8.0)	23 (15.2)
Cesarean section	49 (76.6)	56 (64.4)	105 (69.5)
Intrapartum hemorrhage (g)	941.5 (522.3–1727.0)	690.5 (427.0–1244.3)	821.0 (474.5–1505.8)
Neonatal/placental characteristics			
Birth weight (BW) (g)	2056 (1477–2378)	2310 (1811–2573)	2239 (1620–2524)
Birth height (cm)	44.0 (40.5–47.0)	45.8 (42.0–48.0)	45.0 (41.1–48.0)
Birth head circumstance (cm)	30.6 (28.5–32.0)	31.5 (29.8–33.0)	31.4 (29.4–32.5)
Male	38 (59.4)	36 (41.4)	74 (49.0)
Cord blood pH	7.34 (7.30–7.36)	7.34 (7.31–7.38)	7.34 (7.31–7.37)
Cord blood BE	−2.80 (−4.95–−1.15)	−2.35 (−3.58–−1.20)	−2.50 (−4.20–−1.18)
1-min Apgar score	7.0 (5.0–8.0)	8.0 (6.0–8.0)	8.0 (6.0–8.0)
5-min Apgar score	9.0 (7.0–9.0)	9.0 (7.0–9.0)	9.0 (7.0–9.0)
Placental weight (PW) (g)	442.0 (373.0–569.0)	500.0 (388.5–579.0)	466.5 (384.3–576.0)
Ratio of PW/BW	0.230 (0.201–0.270)	0.227 (0.195–0.268)	0.230 (0.197–0.270)
Histological chorioamnionitis	19 (29.7)	21 (24.1)	40 (26.5)

Data are presented as medians (interquartile ranges) or n (%).

Table 2
Characteristics of the High and Low S100B groups.

	High S100B (*n* = 10)	Low S100B (*n* = 54)	*p*-Value
Maternal characteristics			
Maternal age (years)	33.0 (30.3–35.0)	34.0 (31.0–37.8)	0.426
Primiparity	5 (50.0)	28 (51.9)	1.000
Infertility treatment	3 (30.0)	14 (25.9)	1.000
Body mass index	19.1 (18.0–22.4)	20.7 (19.2–22.3)	0.255
Maternal fever (> 37.5 °C)	4 (40.0)	2 (3.7)	< 0.001
Tocolysis	9 (90.0)	34 (63.0)	0.146
Magnesium sulfate	3 (30.0)	10 (18.5)	0.411
Delivery characteristics			
Gestational weeks at birth (weeks)	33.5 (30.3–35.0)	33.0 (30.0–35.0)	0.794
Labor onset	7 (70.0)	20 (37.0)	< 0.001
Induction of labor	1 (10.0)	7 (13.0)	1.000
Preterm rapture of membrane	3 (30.0)	24 (44.4)	0.498
Fetal tachycardia (> 160 bpm)	1 (10.0)	3 (5.6)	0.502
Non-reassuring fetal status	4 (40.0)	12 (22.2)	0.252
Cesarean section	5 (50.0)	44 (81.5)	< 0.001
Intrapartum hemorrhage (g)	529.5 (474.3.0–799.5)	1062.0 (550.0–1912.8)	0.087
Neonatal/placental characteristics			
Birth weight (BW) (g)	2038 (1479–2189)	2004 (1474–2312)	0.579
Relative birth weight z-score (SD)	−0.29 (−1.00–0.60)	0.30 (−0.17–0.87)	0.128
Birth height (cm)	46.5 (42.5–47.0)	43.8 (39.7–46.0)	0.398
Relative birth height z-score (SD)	0.65 (0.40–0.90)	0.20 (−0.37–1.20)	0.352
Birth head circumstance (cm)	30.6 (28.5–31.0)	30.0 (28.1–32.0)	0.533
Male	8 (80.0)	30 (55.6)	0.181
1-min APGAR score	7.0 (5.3–8.0)	7.0 (5.0–8.0)	0.985
5-min APGAR score	8.0 (7.3–9.0)	8.0 (6.3–9.0)	0.917
Placental weight (PW) (g)	439.0 (362.5–493.0)	436.5 (356.5–556.8)	0.822
Ratio of PW/BW	0.243 (0.235–0.276)	0.230 (0.206–0.272)	0.917
Histological chorioamnionitis	3 (30)	16 (29.6)	1.000

Data are presented as medians (interquartile ranges) or n (%).

Table 3
Cord blood biomarkers of the High and Low S100B groups.

	High S100B (n = 10)	Low S100B (n = 54)	p-Value
Gas analysis			
pH (< 7.27)	4 (40.0)	3 (5.6)	0.009
BE (< −2.7)	8 (80.0)	23 (42.6)	0.041
Pro-inflammatory cytokines			
TNF-α (pg/mL)	27.8 (20.0–44.8)	23.82 (17.7–29.3)	0.185
IL-1β (pg/mL)	0.87 (0.59–2.02)	0.30 (0.13–0.41)	< 0.001
IL-6 (pg/mL)	1.14 (0.64–5.45)	1.03 (0.56–1.37)	0.382
Th1 cytokines			
IFN-γ (pg/mL)	13.2 (11.9–19.2)	12.3 (8.6–16.4)	0.465
IL-2 (> 0.20 pg/mL)	5 (50.0)	14 (25.9)	0.147
IL-12 (> 0.22 pg/mL)	1 (10.0)	14 (25.9)	0.429
Th2 cytokines			
IL-4 (pg/mL)	0.19 (0.14–0.33)	0.14 (0.04–0.14)	0.031
IL-5 (pg/mL)	6.41 (4.87–9.64)	5.53 (3.87–7.60)	0.419
IL-10 (pg/mL)	2.31 (1.50–3.39)	1.90 (1.11–2.64)	0.325
IL-13 (> 0.05 pg/mL)	5 (50.0)	21 (38.9)	0.728
Th17 cytokines			
IL-17 (> 0.19 pg/mL)	7 (70.0)	21 (38.9)	0.090
Growth factors			
IL-7 (> 0.14 pg/mL)	4 (40.0)	23 (42.6)	1.000
GM-CSF (> 0.04 pg/mL)	3 (30.0)	16 (29.6)	1.000
G-CSF (pg/mL)	11.8 (1.51–279.3)	11.5 (4.58–21.63)	0.839
Chemokines			
IL-8 (pg/mL)	11.9 (9.60–158.8)	13.9 (8.40–22.0)	0.882
MCP-1 (pg/mL)	421.8 (317.6–676.7)	277.3 (200.9–461.3)	0.074
MIP-1 β (pg/mL)	65.2 (41.0–119.1)	47.0 (32.2–71.2)	0.163

Data are presented as medians (interquartile ranges) or n (%). pH, Potential of hydrogen; BE, Base excess; TNF-α, Tumor necrosis factor-α; IL, Interleukin; GM-CSF, granulocyte monocyte-colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; IFN-γ, Interferon-γ; MCP-1, monocyte chemotactic protein-1; MIP-1β, macrophage inflammatory protein 1-β.

S100B concentrations in this study, which seems to be inconsistent with the reported findings. A potential reason for this discrepancy could be the temporal difference caused by adopting cord S100B as an outcome. The previous findings showed the relation of the proinflammatory cytokines to clinical neonatal outcomes, but high concentrations of S100B occurred before the development of any clinical signs of brain damage [6]. Moreover, IL-1β and TNF-α are characterized as early response cytokines that are released by rapid induction from diverse stimuli and proinflammatory effects [25]. Interleukin-1β concentrations in this study were in accordance; however, IL-6 levels were much lower than those of the previous studies [1,4] (Table 3). Thus, it could be presumed that we represented phenomena at an earlier phase of fetal brain insults.

Table 4
Associated factors with high S100B by Multivariate logistic regression analysis.

	OR	95% CI	p-Value
Maternal/delivery characteristics			
Maternal fever (> 37.5 °C)	0.471	0.019–11.95	0.648
Labor onset	4.099	0.245–68.68	0.327
Cesarean section	9.913	0.505–194.48	0.131
Cord blood biomarkers			
pH (< 7.27)	4.817	0.379–61.28	0.226
BE (< −2.7)	1.563	0.145–16.85	0.713
IL-1β	6.589	1.126–38.55	0.036
IL-4	0.582	0.031–10.78	0.716

OR, odds ratio; CI, confidence interval; pH, Potential of hydrogen; BE, Base excess; IL, Interleukin.

The IL-4 in the cord blood also showed a positive relationship with high concentrations of S100B (Table 3). Matoba et al. revealed that cord IL-4 concentrations were increased in preterm birth [12]. However, to the best of our knowledge, IL-4 in cord blood has not yet been studied clinically in relation to perinatal brain damage. The difference in the IL-4 levels between the groups is very small in the present study. Therefore, the role of IL-4 in human perinatal period remains unclear.

The pH and BE are routinely measured from the umbilical arterial cord blood at birth. An association between neonatal encephalopathy and birth asphyxia has been established [26]. Several studies also showed a significant association of S100B concentrations to birth asphyxia and HIE [7,27–29]. In these studies, birth asphyxia was defined as the presence of cord pH < 7.0 and/or BE < −12 mmol/L. Our study supports those findings; however, notably, no cases in our study had the asphyxia ranges of cord pH and BE (all our study cases in cord pH > 7.15 and BE > −10 mmol/L) (Fig. 1). This means that the dynamics of cord S100B concentrations cover a wide range of levels of acidemia from normal to severely abnormal.

Our data suggested that S100B levels in cord blood are related to the mode of delivery (Table 2). It has been reported that several markers of brain damage, including S100B in cord blood, are significantly higher in vaginal delivery than in cesarean section [30,31], which is consistent with our finding. Interestingly, S100B concentrations in this study were also affected by the presence of labor onset (Table 2). Taken together, these results could suggest that uterine contraction itself, rather than vaginal delivery, would have an impact on the central nervous system and induce the elevation of S100B.

Several investigators have proposed that maternal fever during labor is associated with an increased risk of encephalopathy and neonatal seizures [32,33], and also increased risk of cerebral palsy both in preterm and term infants [34]. These reports could explain the increase

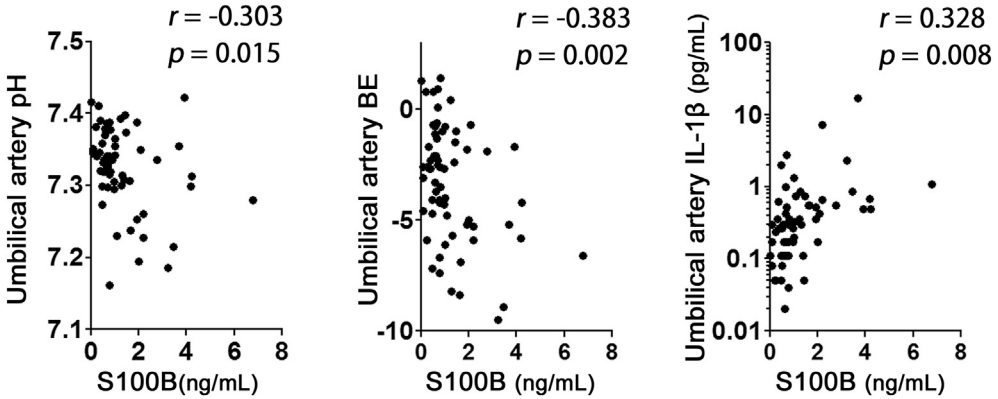

Fig. 1. Dependence of pH, BE, and IL-1β on cord blood S100B.
The umbilical artery pH and levels of BE and IL-1β are strongly associated with cord blood S100B. p and r values for each pair are shown in the respective panels. pH, potential of hydrogen; BE, base excess; IL, Interleukin.

in S100B that was associated with the presence of maternal fever in the present study (Table 2).

Although we speculated the possibility of gender-dependent interactions, no obvious difference was observed in the concentrations of the 17 cytokines. The cord blood S100B were also not affected by the fetus gender, which is consistent with previous findings [8] (Supplemental Table 1).

There are some limitations of the present study. First, this report did not refer to fetuses with pathological conditions, such as gestational diabetes mellitus and preeclampsia. Second, although it is thought to be meaningful to analyze mild brain damage which is not clinically apparent [35], there were few cases of clinically severe brain injury in this study; One case of PVL and four cases of developmental disorders. Although, we classified the five cases collectively as “adverse outcomes cases” and summarized the associations with cord blood S100B and the cytokines, no obvious difference was observed (Supplemental Table 2). However, the present results might be statistically indefinite due to the small sample size, so the results should be interpreted with caution. Further studies in multicenter settings are required to overcome these limitations.

In conclusion, our study reported the association between the levels of 17 cytokines in umbilical cord blood and cord blood S100B levels in preterm infants. S100B could be affected by certain maternal/delivery characteristics and cord blood cytokines, especially IL-1β. These results provide important information regarding the evaluation of cord blood S100B, which could be useful for additional studies on S100B as a biomarker of fetal brain damage, and consequently perinatal brain injury.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.earlhumdev.2019.01.013.

Ethics approval

The study was approved by the institutional review board and ethics committee of Nagoya University Graduate School of Medicine (approval number 20180211), and written informed consent was obtained from all patients.

Code of ethics

The authors ensure that the study was carried out in accordance with the declaration of Helsinki.

Funding sources

There are no external funding sources for the present study.

Author's contributions

N.Y. conducted the experiments, analyzed the data, and wrote the manuscript. K.I. conceived and designed the experiments, analyzed the data, and wrote the manuscript. T.K., R.M., T.N., T.U, Y.M., and F.K. analyzed the data and edited the manuscript. All authors approved the final version.

Declaration of interest

The authors have no competing interests to declare in association with present study.

Acknowledgements

We wish to thank Sachiko Morisaki for her valuable technical support. We would like to thank Editage (www.editage.jp) for English language editing.

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