Protein C pathway in preterm birth with chronic lung disease: Prospective study

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Abstract

Background: Chronic lung disease (CLD) is a major neonatal pulmonary disorder associated with inflammation. Recent studies have shown that protein (P) C anticoagulant pathways, such as those for PC, PS, and thrombomodulin (TM), could be useful indices for reflecting pulmonary injury. However, the involvement of these factors in preterm infants with very low birth weight (VLBW) who have developed CLD remains to be investigated. Here, we investigated whether PC pathway-related factors could predict the development of CLD in preterm infants with VLBW. **Methods:** We collected plasma samples from 26 preterm infants with VLBW (13 each from those with and without CLD) at the time of birth and measured TM, PC, and PS levels in their plasmas. We retrospectively analyzed the relationship between these factors in infants with and without CLD. **Results:** There were significant differences in gestational age, birth weight, Apgar score (5 min), and duration of mechanical ventilation between the CLD and non-CLD groups. No significant differences in the PC and PS levels at birth were observed between the two groups, whereas the TM levels in the CLD group were significantly higher than those in the non-CLD group ($p=0.013$). TM levels correlated with gestational age and duration of mechanical ventilation. However, covariance analysis demonstrated that gestational age was significantly associated with TM levels, and consequently, development of CLD was not associated with TM level at birth. **Conclusion:** TM, PC, and PS levels at birth could not predict the development of CLD in preterm infants with VLBW.

Keywords: mechanical ventilation, inflammation, anticoagulant factor, thrombomodulin, very low birth weight neonates

Introduction

Chronic lung disease (CLD) is one of the most common respiratory diseases in preterm infants who require mechanical ventilation and oxygen therapy because of an imbalance between injury and repair mechanisms in the developing lung. The incidence of CLD increases as the gestational age and/or birth weight of infants decreases [1]. Pulmonary function in infants with CLD presents low lung compliance and lung volumes and elevated pulmonary resistance [2]. CLD is associated with severe neurodevelopmental and growth delay [3-5], resulting in a delay in hospital discharge and the need for supplemental oxygen [6]. Several studies have suggested that infants with CLD show high levels of inflammatory cytokines such as interleukin (IL)-6, IL-8, and tumor necrosis factor α [7,8], indicating that inflammation plays an important role in the pathogenesis of CLD. A previous report suggested that systemic corticosteroid treatment could not be useful for preventing CLD in preterm infants [9]. The use of inhaled glucocorticoids in preterm infants appears to have beneficial effects for the prevention or treatment of CLD. However, a large clinical trial demonstrated that inhaled glucocorticoids did not improve the rates of neurodevelopmental disability and that the mortality rates were higher in patients who received inhaled glucocorticoids than in those who did not [10]. Consequently, the frequency of CLD is still considerable, although advances in neonatal care have contributed to improved survival and decreased morbidity [11].

Thrombomodulin (TM) is a thrombin-binding anticoagulant cofactor that functions as a cell surface receptor [12-14]. Protein C (PC) and protein S (PS) are two vitamin K-dependent plasma proteins that act as natural anticoagulants [15]. TM is a cofactor for thrombin-catalyzed activation of PC, and activated PC (APC) inactivates coagulation activated factor V (FVa) and factor VIII (FVIIIa), leading to the inhibition of thrombin activity [16]. PS is a cofactor for APC and APC, together with PS, inhibits coagulation by degrading FVIIIa and FVa [17]. Overall, the PC anticoagulant pathways, such as those for TM, PC, and PS, play a crucial role in modulating the coagulation potential.

The association between inflammation and TM, PC, and PS has recently been documented [18-25]. Severe sepsis is associated with low APC levels and increased mortality [21]. Human PS protects against lipopolysaccharide-induced acute lung injury [23]. Another study demonstrated that adult patients with acute lung injury had increased TM levels and decreased plasma PC levels [24]. In addition, TM levels in plasma reflect pulmonary endothelial damage in preterm infants with respiratory distress syndrome (RDS) [25]. These reports indicated that TM, PC, and PS have the potential to reflect inflammatory response or lung injury. CLD could also be involved in pulmonary inflammation, but the association between these anticoagulant factors and CLD in preterm infants remains to be investigated.

We hypothesized that TM, PC, and PS levels in the plasma of preterm infants at the time of delivery might be useful predictors for the development of CLD. In the present study, we examined the TM, PC,

and PS at the time of birth in preterm infants with and without CLD and also determined whether these PC-related anticoagulant factors could be beneficial for predicting CLD in very low birth weight (VLBW) infants.

Materials and Methods

Ethics - This study was approved by the Medical Research Ethics Committee of Nara Medical University (No. 1326), and blood samples were collected after informed consent was obtained from patients in accordance with the ethical guidelines of our university.

***Inclusion and exclusion criteria and definition*:** A patient flow of all eligible infants for this study is shown in Supplemental Figure 1. Twenty-six infants weighing less than 1,500 g (VLBW infants) were enrolled. Thirteen preterm infants developed CLD (CLD group) and 13 preterm infants did not develop CLD (non-CLD group). Fifty-three preterm infants were excluded, because we could not draw enough blood volumes from them. One infant with complications of severe thrombocytopenia and bleeding tendency was excluded. In the present study, severe thrombocytopenia as the patient who had platelet counts of less than $10 \times 10^3/\mu L$ and presented bleeding complications was defined. CLD was defined as requiring supplemental oxygen at 36 weeks' postmenstrual age. RDS was diagnosed based on clinical symptoms, blood gases, and chest radiograph findings. The diagnosis of CLD and RDS was judged by plural physicians specialized in Neonatal Intensive Care Unit at our hospital.

***Blood samples*:** Blood samples were collected from all 26 VLBW infants with and without CLD. Blood samples were obtained from patients within 1 hour of birth. Whole blood samples were collected in plastic tubes containing 3.2 % sodium citrate at a ratio of 9:1 (Fuso Pharmaceutical Industries, Osaka, Japan). Platelet-poor plasma was collected after centrifugation of the citrated whole blood for 15 min at 1,500 $\times g$. All plasma samples were stored at −80 °C and thawed at 37 °C immediately prior to the assays.

***Measurement of TM, PC, and PS levels in plasma*:** The plasma concentration of TM was measured using the SimpleStep ELISA® kit (Abcam, Cambridge, UK), according to the manufacturer's instructions. The plates were read at 450 nm using an automated microplate reader (Multiskan®; Thermo Fisher Scientific). Tests for PC and PS activity were performed on the CS2400i® (Sysmex) analyzer using the HEMOCLOT™ kit. These TM, PC, and PS values in our patients were determined when 5-10 plasma samples had been collected.

Data analysis - Data analysis was performed using EZR [26] for R. More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics. All data are presented as median and interquartile ranges (IQR). Significant differences were determined using the Mann–Whitney *U*-test and Fisher's exact test. Analysis of covariance (ANCOVA) was used to

assess the statistical significance between the thrombomodulin level and CLD or gestational age. ANCOVA was performed using JMP®10 (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $P < 0.05$.

Results

***Enrolled patients*:** From October 2016 to January 2019, 91 VLBW infants were born in our NICU, and 11 cases of them died before hospital discharge. Eight of 11 demised cases died within 1 week after birth and 3 of them survived after 36 weeks of gestation. We could not take their blood samples due to poor general status. Of the 80 surviving cases, 26 preterm infants were enrolled in the present study as described above (Supplemental Figure 1). There were no differences in clinical characteristics between the excluded subjects (n = 53) and research subjects (n = 26) (Supplemental Table 1), indicating that our research had no biases associated with the research subjects.

***Clinical characteristics of enrolled patients*:** A total of 26 infants with a median gestational age of 28.2 (IQR; 26.8, 31) weeks and a median birth weight of 997 (640, 1,229) g were enrolled. The clinical and maternal characteristics of the VLBW infants who developed CLD (n = 13; CLD group) and those who did not (n = 13; non-CLD group) are summarized in **Table 1**. The median gestational age and birth weight in the CLD group were shorter and lower than those in the non-CLD group, respectively ($p < 0.01$). The number of patients who received surfactant therapy and the periods of mechanical ventilation and oxygen therapy in the CLD group were greater and longer than those in the non-CLD group, respectively ($p < 0.01$). In addition, the Apgar score in the CLD group was lower than that in the non-CLD group ($p < 0.05$). We found no significant difference between the two groups in terms of the number of patients with small-for-gestational age, use of antenatal corticosteroid, complications of hypertensive disorders of pregnancy, and chorioamnionitis. Laboratory data revealed that blood gas, C-reactive protein, and IgM values were not significantly different between the two groups.

***Evaluation of PC levels in preterm infants*:** We first focused on the PC activity level in VLBW infants. The median level of PC activity in all infants was 9.2 (IQR; 8, 13.9 %). We evaluated whether the PC activity value in both groups at the time of birth was useful for predicting the development of CLD (**Fig. 1**). However, no significant difference was observed in the PC levels between the two groups. Furthermore, PC values had little correlation with gestational age, birth weight, Apgar score, and duration of mechanical ventilation (data not shown). These findings indicated that PC activity level at birth was not useful in discriminating between VLBW infants with CLD and those without.

***PS level in preterm infants*:** We next assessed the PS activity level in both groups. The median level of PS activity in all infants was 15.5 % (IQR; 12.8, 19.5 %). There was no significant difference in the PS activity levels between the two groups (**Fig. 2A**). However, a correlation was observed between PS and Apgar score (at 5 min) or the duration for requiring mechanical ventilation ($r = 0.54$ or -0.44,

respectively). We also found a correlation between PS level and gestational age or birth weight (r = 0.49 or 0.67, respectively) (**Fig. 2B**). These results supported the fact that PS activity level at birth did not appear to reflect VLBW infants with CLD.

TM level in preterm infants: We further investigated the relationship between TM antigen levels in both groups. The median value of TM antigen in all infants was 3,729 (IQR; 3,226, 4,372) pg/mL. No significant difference was observed between TM level and birth weight or Apgar score at 5 min. In contrast, TM levels in infants with CLD were significantly higher than in those without CLD ($p = 0.013$) (**Fig. 3A**). However, we found that TM levels were associated with gestational age and duration of mechanical ventilation (r = −0.64 and 0.49, respectively) (**Fig. 3B**). After adjustment for gestational age, covariance analysis demonstrated that TM levels at birth were not correlated with the development of CLD. In contrast, gestational age was associated with TM after adjustment for CLD (**Table 2**). Overall, TM at birth did not appear to be a useful index for predicting the development of CLD in VLBW infants.

Discussion

We speculated that PC systems such those of PC, PS, and TM could be useful for detecting CLD status in infants. In this context, we investigated these anticoagulant factors at birth in VLBW infants (<1,500 g). To our knowledge, this is the first study to assess these factors as predictors of CLD in infants. Our findings suggest that TM values at birth were significantly higher in infants with CLD than in those without, but that the PC pathway was not useful for predicting CLD infants.

As mentioned above, the median PC level in our enrolled infants was 9.2 %. An earlier study reported that the mean PC value in healthy preterm infants (30 to 36 weeks gestation) was 28 % [27], indicating that PC values in our infants were markedly lower. The synthesis of PC as a single chain protein occurs in the liver [28]; therefore, the lower PC level could be attributed to vitamin K-dependent coagulation factor and an immature liver in preterm infants. Our data suggested that the PC value in our cases was not correlated with gestational age, birth weight, Apgar score, and duration of mechanical ventilation. A similar report suggested that the PC value could be a useful biological marker for adult patients who developed acute lung injury or acute respiratory distress syndrome [24]. The discrepancy between these results remains unclear; however, the PC level in our VLBW infants may not have responded to inflammatory reactions due to an immature liver. Overall, the PC measurements after birth did not provide beneficial information in the CLD infants.

With regard to PS, the median PS level in our infants was 15.5 %. The mean PS value in healthy preterm infants (30 to 36 weeks gestation) was 26 % [27], indicating that the PS and PC levels in our infants were also significantly lower than those in previous studies. PS is also synthesized in the liver [29], and a lower PS level could be related to vitamin K-dependent coagulation factor and an immature liver in

our cases. Unexpectedly, PS was associated with gestational age, birth weight, Apgar score, and duration of mechanical ventilation. The reason for the correlation between PS and these factors remains unclear. To our knowledge, there is little information available regarding the association between PS and preterm infants. However, a previous study demonstrated an inverse correlation between gestational age in pregnant women and free PS level [30]. It may be possible that the health conditions of pregnant women are associated with the PS level and clinical conditions in preterm infants.

An earlier study demonstrated that TM concentrations in the lung and placenta were higher than those in several human tissues, as measured by radioimmunoassay [31]. In addition, several studies have suggested that TM levels could be useful as a biological marker of pulmonary damage [24,25]. These results suggest that TM may be closely related to pulmonary function or injury. As expected, our findings showed that TM levels in the CLD group were significantly higher than those in the non-CLD group. Although a correlation between TM level and gestational age or the duration required for mechanical ventilation was also observed, covariance analysis confirmed that TM levels in our infants were associated only with gestational age. Nako *et al.* demonstrated that birth weight contributed significantly to the TM concentration in plasma [32]. The discrepancy between our results and those of previous studies may be attributed to the sample population. Their analyses of TM levels involved all infants, including full-term infants [32]. Specifically, half of their infants were full-term, indicating that their results may not reflect the correlation between TM levels and preterm infants. Regarding pulmonary damage, no significant difference in plasma TM concentration was observed between VLBW infants who did or did not require mechanical ventilation [32], indicating that their results were not consistent with ours. The mean gestational ages of their preterm infants who did or did not require mechanical ventilation were 28.4 and 30.7 weeks, respectively, showing little difference in the gestational ages. The median gestational ages of our cases that did or did not require mechanical ventilation were 26.7 and 31.0 weeks, respectively. Therefore, it could be that the difference in gestational age contributed to the discrepancy in the results. The incidence of CLD in infants whose gestational age was ≤ 28 weeks has been approximately 40 % over the last few decades [11]; therefore, our findings may accurately reflect the association between TM and preterm infants requiring mechanical ventilation.

The limitations of this study must be discussed. First, only a limited number of cases were included because taking enough blood volumes without hemolysis and coagulation in very or extremely low birth weight infants required extensive skills and enough medical workforces. Also, CLD are usually complicated in preterm infants born under 28 weeks of gestational age and age-matched CLD and non-CLD groups were difficult to be available for comparisons. Second, we did not investigate the PC anticoagulant pathway after birth in our infants. Third, we could not evaluate the association between

the PC anticoagulant pathway and lung inflammation, as few infants with high levels of serum IgM, severe chorioamnionitis, and severe funisitis at birth were observed (Table 1).

Nevertheless, we believe that our study is significant because there have been no research reports of the PC pathway system in, especially, very or extremely low birth infants. Our results suggest that TM, PC, and PS levels at birth are unlikely to be optimal for predicting the development of CLD in VLBW infants. Further studies on useful biological markers of CLD infants should be conducted.

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Disclosure Statement

All authors have no conflicts of interest.

Author Contributions

TK performed the experiments, created the figure, and wrote the paper; **YN** performed the experiments, analyzed the data, wrote the paper, and approved the fina! version to be published; **YU, TN, HT, YT,** and **EN** provided clinical supported; **YT** interpreted the data and supervised the research; **TN** supervised the research and supported the study; **KN** designed the research, interpreted the data, wrote the paper, and edited the manuscript. All authors read and approved the final manuscript.

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Table 1. Clinical characteristics and laboratory data in the enrolled VLBW infants

	CLD (n=13)	Non-CLD (n=13)	*P* value
Male/Female (n)	7 / 6	7 / 6	*N.S.*
Gestational age (week)	26.8 (24.9–28.4)	30.9 (27.7–34.3)	<0.01
Birth weight (g)	703 (482–1,064)	1,191 (755–1,456)	<0.01
SGA (n)	7	5	*N.S.*
HDP (n)	5	4	*N.S.*
Antenatal corticosteroid (n)	8	5	*N.S.*
CAM (n)	1	3	*N.S.*
APGAR score (at 1 / 5 min)	4.2 / 6.4	6.6 / 8.7	<0.05
Use of surfactant (n)	13	3	<0.01
Duration; Mechanical ventilation (day)	79.9 (33–201)	7.9 (0–41)	<0.01
Duration; Oxygen therapy (day)	96.0 (59–206)	12.3 (1–48)	<0.01
Laboratory data			
pH	7.224 (7.109–7.399)	7.211 (7.139–7.402)	*N.S.*
HCO_3 (mEq/L)	21.6 (14.1–26.6)	23.3 (17.1–31.4)	*N.S.*
BE (mEq/L)	-5.3 (-13.4– -0.4)	-5.3 (-12.3– +2.8)	*N.S.*
CRP (mg/dL)	0 (0–0.04)	0 (0–0.35)	*N.S.*
IgM (mg/dL)	4.3 (2.3–10)	11.5 (1.5–63.8)	*N.S.*
Maternal CRP (mg/dL)	0.59 (0.20–2.09)	0.90 (0.04–3.50)	*N.S.*

Data are presented as median and interquartile range (IQR). VLBW, very low birth weight; CLD, chronic lung disease; NS, not significant; SGA, small-for-gestational age; HDP, hypertensive disorders of pregnancy; CAM, chorioamnionitis; BE, base excess: CRP, C-reactive protein

Table 2. Covariance analysis (ANCOVA) between thrombomodulin and gestational age or CLD infants

Thrombomodulin	*F* value	*P* values
CLD	0.19	*N.S.*
Gestational age	4.78	<0.05

CLD, chronic lung disease

Figure Legends

Figure 1. Protein C (PC) levels at birth in chronic lung disease (CLD) and non-CLD infants - The PC values at birth were obtained from CLD infants (n = 13) and non-CLD infants (n = 13). Each box plot shows the interquartile range (IQR) with median values (horizontal line). Significant differences among these groups were considered as $p < 0.05$.

Figure 2. Protein S (PS) levels at birth in chronic lung disease (CLD) and non-CLD infants - *Panel* **(A)** The PS values at birth were obtained from CLD infants (n = 13) and non-CLD infants (n = 13). Each box plot shows the interquartile range (IQR) with median values (horizontal line). $p < 0.05$ indicates significant differences among these groups. *Panel* **(B)** Correlation between PS values and Apgar score at 5 min, mechanical ventilation, gestational age or birth weight in all infants. The solid line indicates the correlation coefficient (R).

Figure 3. Thrombomodulin levels at birth in chronic lung disease (CLD) and non-CLD infants - *Panel* **(A)** Thrombomodulin (TM) values at birth were obtained from CLD infants (n = 13) and non-CLD infants (n = 13). Each box plot shows the interquartile range (IQR) with median values (horizontal line). Significant differences among these groups were considered as $p < 0.05$. *Panel* **(B)** Correlation between TM values and gestational age or mechanical ventilation in all infants. The solid line represents the correlation coefficient (R).

Supplemental Figure 1. Patient flow of all eligible preterm infants - Total of 91 infants with very low birth weight (VLBW) less than 1,500g was born in our hospital. The survival cases of VLBW infants were 80 and 26 infants of them were research subjects. We could not take enough blood samples from the excluded 53 preterm infants. One infant with severe thrombocytopenia and bleeding tendency was also excluded. Thirteen out of research subjects had chronic lung disease (CLD) and the others did not have CLD.

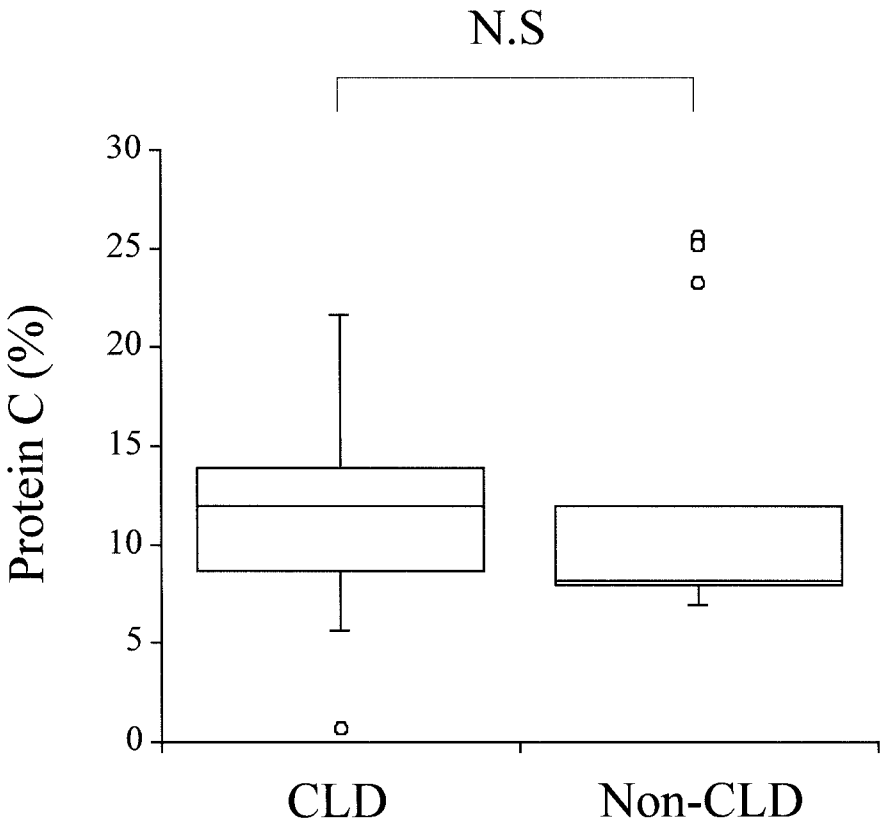
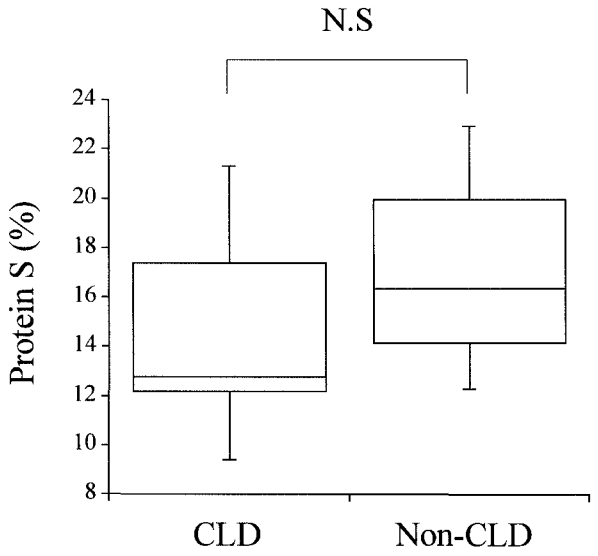


Figure 1

(A)


N.S
Protein S (%)
24
22
20
18
16
14
12
10
8
CLD
Non-CLD

(B)

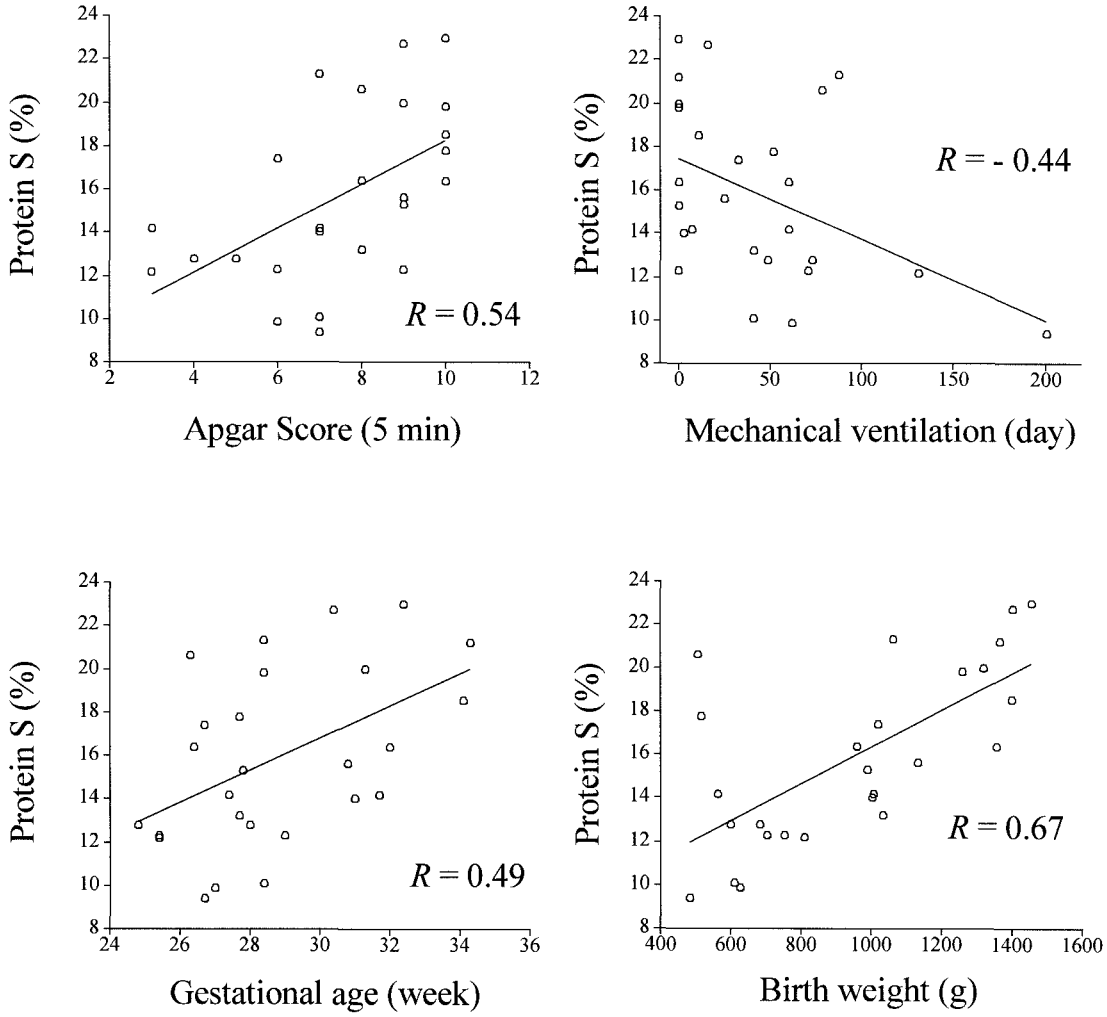
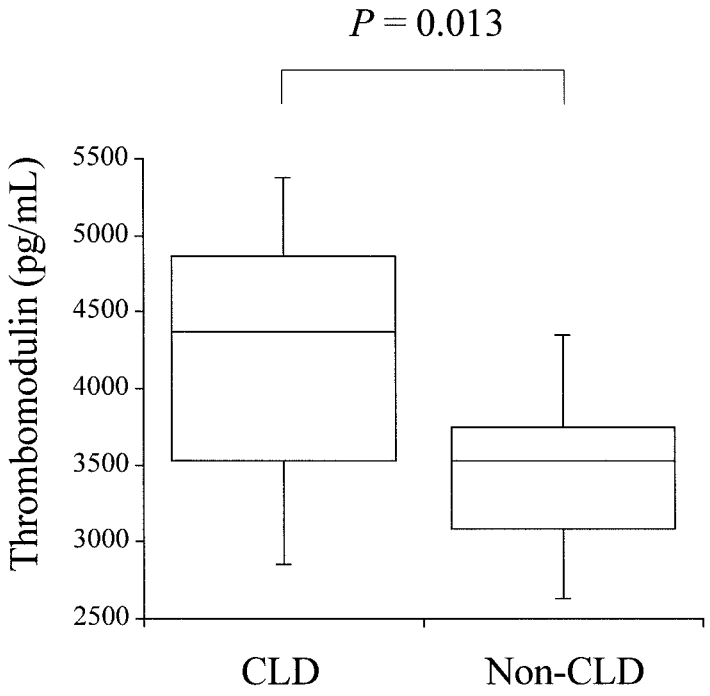

Protein S (%)
R = 0.54
Apgar Score (5 min)
R = - 0.44
Mechanical ventilation (day)
R = 0.49
Gestational age (week)
R = 0.67
Birth weight (g)

Figure 2

(A)


P = 0.013
Thrombomodulin (pg/mL)
5500
5000
4500
4000
3500
3000
2500
CLD
Non-CLD

(B)

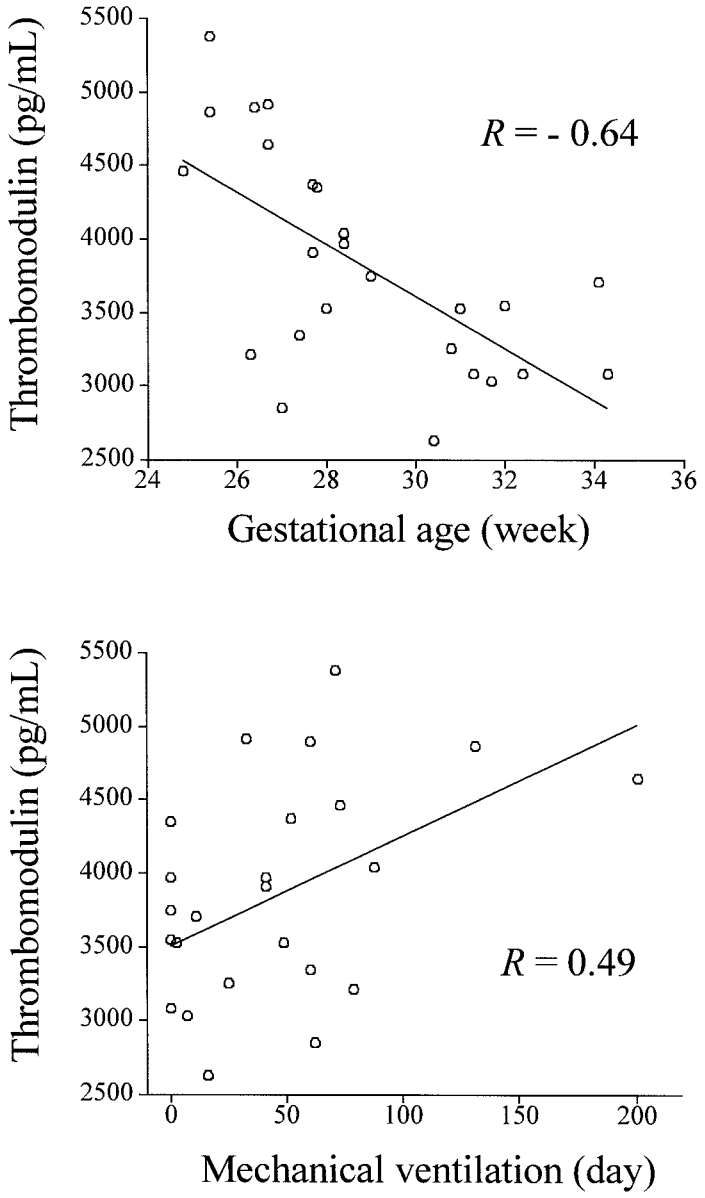
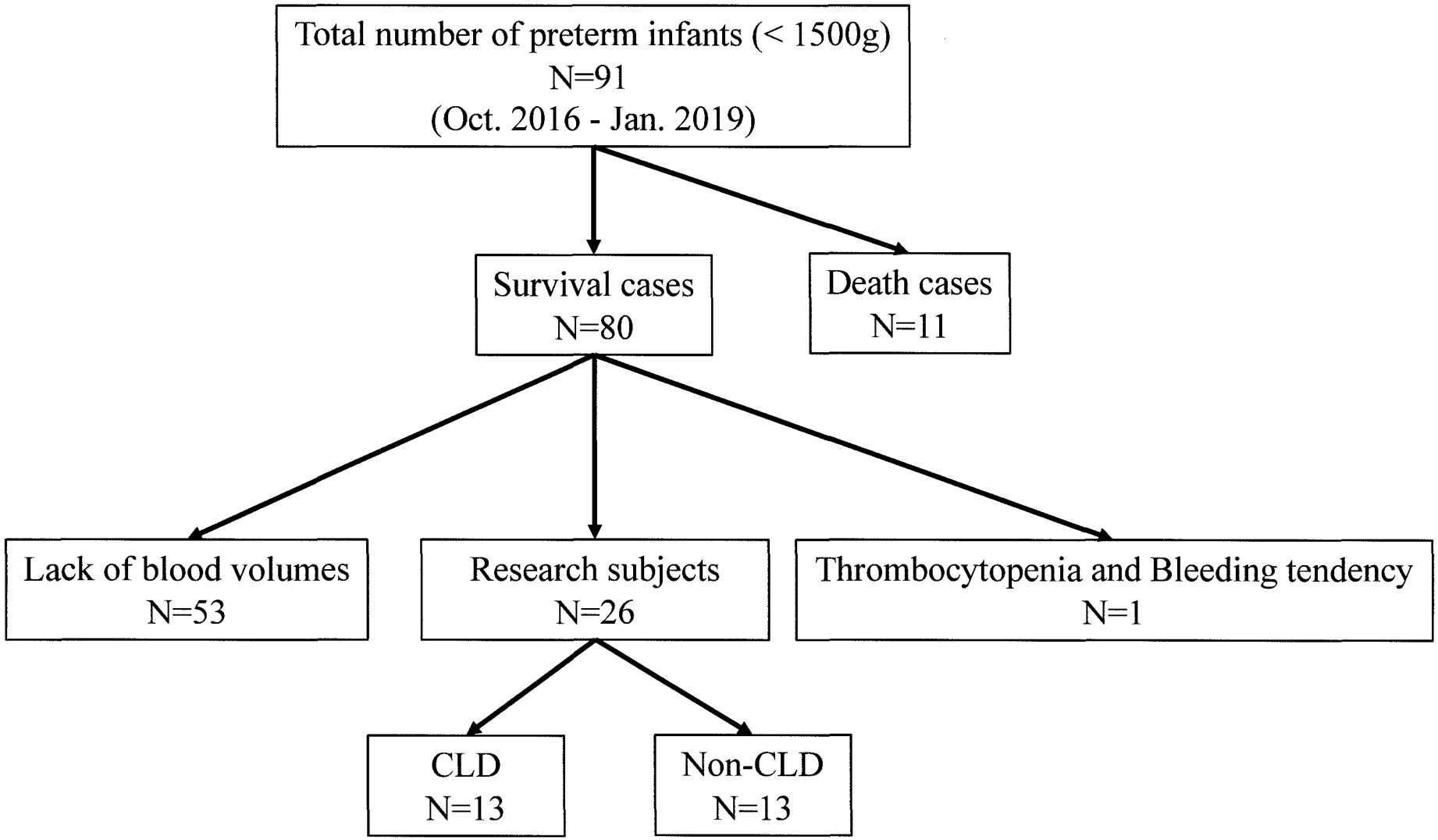

Thrombomodulin (pg/mL)
R = - 0.64
5500
5000
4500
4000
3500
3000
2500
24
26
28
30
32
34
36
Gestational age (week)
Thrombomodulin (pg/mL)
R = 0.49
5500
5000
4500
4000
3500
3000
2500
0
50
100
150
200
Mechanical ventilation (day)

Figure 3



Supplemental Figure 1