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Increased Levels of Interleukin-6 in Tracheal Aspirate Fluid Are Indicative of Fetal Inflammation in Ventilated Extremely Low Gestational Age Newborns

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OBJECTIVE: To determine whether increased serum and/or tracheal aspirate fluid (TAF) levels of IL-6 at birth are associated with fetal inflammation in ventilated extremely low gestational age newborns (ELGAN). METHOD: A total of 36 ELGAN who required mechanical ventilation were enrolled in this study. The patients were classified into two groups: 19 infants who displayed histological evidence of funisitis, which is a marker of fetal inflammation, (funisitis group) and 17 infants without funisitis (comparison group). TAF samples were obtained during routine endotracheal suctioning performed within 2 hours of birth. RESULTS: The funisitis group exhibited significantly higher TAF IL-6 levels than the comparison group (2245 vs. 113 pg/mg total protein; p<0.001). The serum IL-6 levels of the funisitis group were also significantly elevated compared with those of the comparison group (median: 737 vs. 136 pg/mL, p=0.017). Receiver operating characteristic curve analysis of the association between IL-6 levels and the presence of funisitis revealed that the TAF IL-6 concentration had a higher area under the curve (0.947) than the serum IL-6 concentration (0.719). At a cut-off value of 216 pg/mg total protein, the TAF IL-6 level exhibited sensitivity and specificity values of 94.7% and 86.7%, respectively, for detecting funisitis. CONCLUSION: Elevated TAF IL-6 levels at birth are strongly associated with funisitis. The TAF IL-6 concentration is a useful marker for detecting fetal inflammation in ventilated ELGAN.

INTRODUCTION

Histological chorioamnionitis and funisitis are frequently observed in the preterm placentas of women that experience preterm labor or premature rupture of membranes and are associated with positive amniotic fluid and chorioamniotic space cultures (9). Funisitis is closely associated with antenatal exposure to inflammation, which contributes to worsening pulmonary and neurological function, as well as other organ conditions, in extremely premature infants (6).

In histological chorioamnionitis and funisitis, the presence of fetal signs of inflammation, such as elevated cord blood interleukin-6 (IL-6) levels, is considered to represent the more serious end of the continuum (2, 7). Several studies have attempted to develop sensitive and specific diagnostic markers for histological chorioamnionitis and funisitis by analyzing maternal blood, amniotic fluid, and cord blood. High concentrations of inflammatory markers, including pro-inflammatory cytokines (IL-6, IL-8, IL-1β, and tumor necrosis factor (TNF)-α), have been detected in amniotic fluid and cord blood in the presence of placental histological inflammation (2, 5, 7, 12). To date, however, there have only been a few reports about the association between placental histology and the serum and tracheal aspirate fluid (TAF) IL-6 levels of newborns (1, 3), especially in preterm infants requiring mechanical ventilation, who are at greater risk of neonatal infection and bronchopulmonary dysplasia (BPD).

In this study, we hypothesized that the serum and/or TAF IL-6 levels of ventilated premature infants could be useful markers of fetal inflammation. Accordingly, we aimed to determine whether increased serum and/or TAF IL-6 levels at birth are associated with funisitis in ventilated extremely low gestational age newborns (ELGAN).

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METHODS

1) Study population and study design

ELGAN (gestational age <28 weeks) born at the neonatal intensive care unit of our hospital between July and August 2012 were enrolled in this study. Serum and TAF samples were obtained from the ventilated ELGAN within 2 hours of birth. Informed consent was waived because of the retrospective nature of the data collection. Instead, we informed the public about these experiments via our website. This retrospective study was approved by the ethical committee of Hyogo Prefectural Kobe Children's Hospital.

2) Definition of funisitis

The infants' umbilical cords were analyzed for histological markers of inflammation by a pathologist. Funisitis; i.e., chorioamnionitis with fetal involvement, was diagnosed based on the presence of neutrophil infiltration into the umbilical vessel wall or Wharton's jelly (1).

3) Sample collection and IL-6 assay

Blood and TAF samples were collected within 2 hours of birth. The TAF samples were obtained during routine endotracheal suctioning performed prior to surfactant treatment. The suction tube was flushed with 0.5 mL saline, and the resultant sample was collected in a catheter (11). The levels of IL-6 in the serum and TAF diluent samples were measured with a chemiluminescent enzyme immunoassay (Fujirebio Inc., Tokyo, Japan) within 2 hours of sampling. The total protein concentration of the TAF diluent was verified using a QUICK RUN analyzer (Wako Chemicals, Osaka, Japan). TAF IL-6 levels are often reported as ratios relative to the total protein content of the TAF (4, 11); however, the IL-6 levels in amniotic fluid have been reported as absolute values (5). Therefore, we express IL-6 values as both absolute (pg/ml) and corrected values (pg/mg total protein) to allow comparisons with previous reports.

4) Statistical analysis

The collected data are presented as mean ± standard deviation or median (interquartile range) values. Categorical data were analyzed with the two-tailed $\chi 2$ test or Fisher's exact test where applicable. Continuous data were analyzed using the Student's t-test or Mann-Whitney U test. P-values of <0.05 were considered significant. Receiver operating characteristic (ROC) curves were generated, and the area under the curve (AUC) was analyzed. Pairwise comparisons were performed using SPSS 21.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

1) Patient characteristics

Thirty-six infants with a gestational age of <28 weeks were born during the study period. All of these infants were intubated at birth due to respiratory distress, and hence, were included in the present study. The patients were classified into two groups: 19 infants with funisitis (funisitis group) and 17 infants without funisitis (comparison group).

Table I shows their perinatal characteristics. There were no significant differences in gestational age; birth weight; Z-score; or the frequency of singletons, antenatal steroid use, pregnancy-induced hypertension, gestational diabetic mellitus, or cesarean section between the two groups. The frequency of severe respiratory distress syndrome (RDS; Bomsel grade 3 or 4) was significantly lower in the funisitis group than in the comparison group.

Table I. Patients' characteristics

	Funisitis (n=19)	Comparison (n=17)	P
Gestational age (wks)	25.0 ± 1.5	25.7 ± 1.5	0.171
Birth weight (g)	715 ± 175	743 ± 198	0.655
Z-score	-0.30 ± 0.86	-0.61 ± 1.69	0.502
Antenatal steroids	6 (32%)	10 (59%)	0.101
Singletons	17 (89%)	13 (76%)	0.296
Pregnancy-induced hypertension	0 (0%)	3 (18%)	0.056
Gestational diabetes mellitus	0 (0%)	0 (0%)	1.000
Cesarean section	11 (58%)	14 (82%)	0.112
HC: Blanc grade 0-1	0 (0%)	1 (6%)	0.284
HC: Blanc grade 2	3 (16%)	14 (82%)	<0.001
HC: Blanc grade 3	16 (84%)	2 (12%)	<0.001
RDS	18 (95%)	16 (94%)	0.935
Severe RDS (grade 3 or 4)	8 (42%)	13 (76%)	0.037
Interval between birth and blood sample collection (min)	100 (85-107)	80 (74-108)	0.157
Interval between birth and TAF sample collection (min)	35 (28-50)	39 (34-50)	0.880

Data are presented as mean ± SD, n (%), or median (interquartile range) values.
HC, histological chorioamnionitis; RDS, respiratory distress syndrome; TAF, tracheal aspirate fluid

2) Serum IL-6 and TAF IL-6 levels

Blood samples were collected via an umbilical venous or arterial line (median interval between birth and sample collection: 98 min, interquartile range: 79-108 min), and TAF samples were obtained during routine endotracheal suctioning performed prior to surfactant treatment (median interval between birth and sample collection: 38 min, interquartile range: 28-50 min). No significant differences in the length of the intervals between birth and the collection of the blood or TAF samples were observed between the two groups (Table 1). Blood samples could not be obtained from 1 infant, and TAF samples could not be obtained in another case. The serum and TAF IL-6 levels of the ventilated ELGAN are shown in Figure 1.

The funisitis group displayed significantly higher serum IL-6 levels than the comparison group (737 (162-4340) pg/mL vs. 136 (70-256) pg/mL; p=0.017). The absolute and corrected TAF IL-6 values of the funisitis group were also significantly higher than those of the comparison group (absolute values: 733 (239-6433) pg/mL vs. 30 (13-75) pg/mL, p<0.001; corrected values: 2245 (694-9032) pg/mg total protein vs. 113 (57-179) pg/mg total protein, p<0.001). No significant difference in the total protein concentration of the TAF was observed between the funisitis and comparison groups (41.3 (36.9-92.5) mg/dl vs. 32.6 (3.4-246.8) mg/dl; p=0.250). In addition, further analysis revealed that there was no significant difference in this parameter between the infants with and without severe RDS (53.4 (28.5-172.1) mg/dl vs. 38.2 (21.7-51.8) mg/dl; p=0.077).

3) ROC curve analysis

The results of the ROC curve analysis of the utility of serum IL-6 and TAF IL-6 levels for detecting funisitis are shown in Figure 2.

The corrected TAF IL-6 level was found to be the strongest predictor of funisitis, as it displayed an AUC of 0.947, whereas the absolute TAF IL-6 level and serum IL-6 level exhibited AUC of 0.912 and 0.719, respectively. The corrected TAF IL-6 level displayed sensitivity and specificity values of 94.7% and 86.7%, respectively, for predicting funisitis at a cut-off value of 216 pg/mg total protein, while the absolute TAF IL-6 level demonstrated sensitivity and specificity values of 89.5% and 86.7%, respectively, at a cut-off value of 110 pg/ml (Table II).

Table II. Ability of IL-6 concentrations to predict funisitis

	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
C-TAF IL-6	216 pg/mg total protein	94.7	86.7	95	100
A-TAF IL-6	110 pg/ml	89.5	86.7	89.4	92.9
Serum IL-6	628 pg/ml	52.6	100	100	62.5

C, corrected; A, absolute; IL, interleukin; TAF, tracheal aspirate fluid; PPV, positive predictive value; NPV, negative predictive value

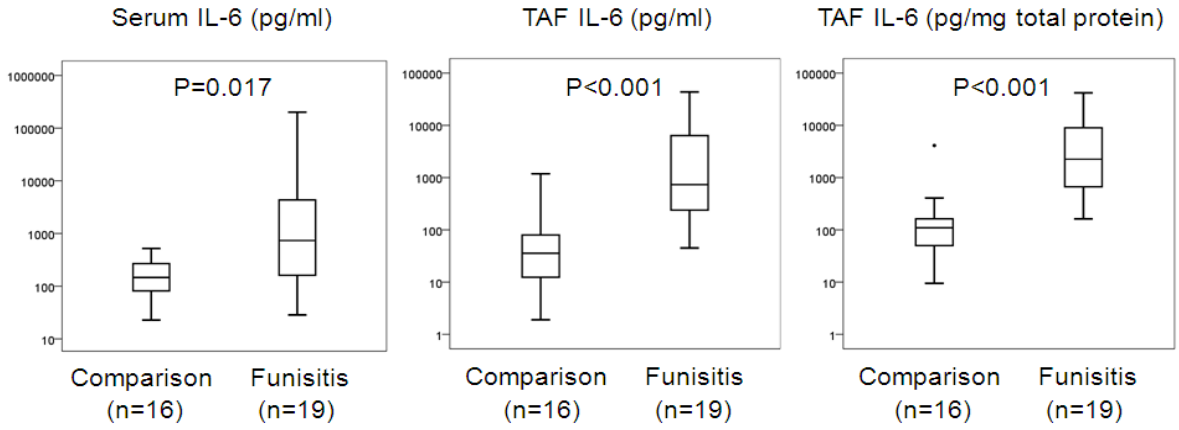

Figure 1. Comparison of the serum IL-6 and TAF IL-6 levels of the comparison and funisitis groups
Box plot: The lower and upper boundaries of the box indicate the 25th and 75th percentiles, respectively; the line through the center of the box represents the median value; and the lower and upper error bars indicate the 5th and 95th percentiles, respectively. The funisitis group displayed significantly higher serum IL-6 levels than the comparison group (p=0.017). The absolute and corrected TAF IL-6 levels of the funisitis group were also significantly higher than those of the comparison group (p<0.001).

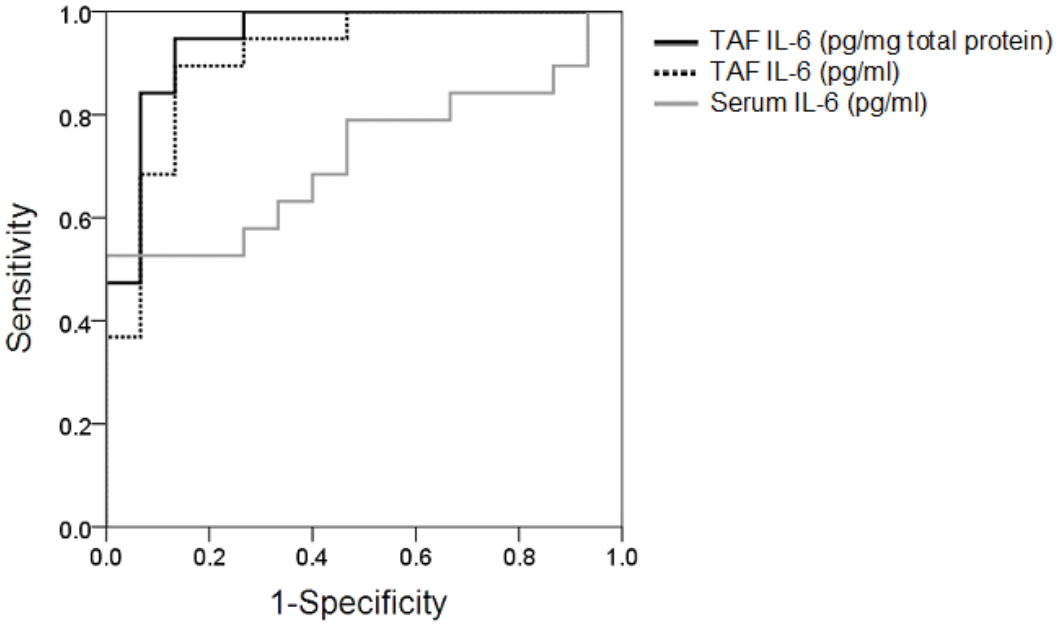

Figure 2. ROC curve analysis of the utility of serum IL-6 and TAF IL-6 levels for detecting funisitis
The corrected TAF IL-6 level was the strongest predictor of funisitis, with an AUC of 0.947, whereas the absolute TAF IL-6 level and serum IL-6 level exhibited AUC of 0.912 and 0.719, respectively.

DISCUSSION

In this study, we demonstrated that ventilated ELGAN with funisitis exhibited significantly higher serum and TAF IL-6 levels at birth than those without funisitis. Moreover, we found that elevated TAF IL-6 levels were more strongly associated with funisitis than elevated serum IL-6 levels.

TAF and bronchoalveolar lavage (BAL) have been used to characterize the concentrations of inflammatory mediators in many lung diseases. For example, some reports have demonstrated increased levels of proinflammatory cytokines in the TAF of ventilated preterm infants who subsequently developed BPD (3). Surprisingly, however, only a few reports have examined the associations between TAF cytokine levels at birth and placental inflammation (1, 3, 4). This can be partly explained by the fact that obtaining TAF or BAL samples involves an invasive procedure in very premature infants. In addition, Aghai studied the association

between histological chorioamnionitis and the IL-6 levels of TAF obtained at 48 hours after birth and found that there was no significant difference between the TAF IL-6 levels of the infants with and without histological chorioamnionitis (1). In our study, we obtained TAF within 2 hours of birth during routine endotracheal suctioning performed in preparation for surfactant administration. Our results demonstrated a clear association between funisitis and the TAF IL-6 level within 2 hours of birth.

This is the first study to compare the utility of TAF IL-6 levels for detecting fetal inflammation with that of serum IL-6 levels. We used serum samples that were obtained after birth, rather than cord blood, so that the comparisons between the serum and TAF IL-6 levels were based on samples that were obtained almost simultaneously. Our data demonstrated that the TAF IL-6 concentration is a more sensitive marker of fetal inflammation than the serum IL-6 level. One possible explanation for this finding is that cytokines are cleared from the lungs more slowly than from the blood. This is supported by the fact that the plasma IL-6 levels of infants with chorioamnionitis were markedly decreased by the first day of life (8). Another explanation is that activated alveolar macrophages in the lungs produce large amounts of inflammatory cytokines. In fact, Schmidt et al. detected increased macrophage infiltration and increased expression of pro-inflammatory cytokines in the lungs of fetuses with chorioamnionitis (10). Thus, studies examining the rapid changes in cytokine levels that occur after birth are required.

Our study had certain limitations. First, we did not collect any data on the TAF of non-intubated infants, which is why we focused on ELGAN requiring mechanical ventilation, who are at greater risk of neonatal infection and BPD. Second, we could not compare TAF and serum IL-6 levels between infants with and without histological chorioamnionitis because of the high frequency of histological chorioamnionitis at our hospital. Thus, studies of the correlations between the severity of chorioamnionitis or funisitis and cytokine levels would be useful. Third, we only measured the serum and TAF levels of IL-6. Whilst multiplex assay kits that can analyze the levels of multiple cytokines in small sample volumes are available, such examinations are time and labor-intensive, and hence, are not ideal for clinicians. On the other hand, IL-6 is a widely used inflammatory marker (2, 7), and it is possible to rapidly analyze IL-6 levels using enzyme immunoassay kits at our institution. Thus, we used IL-6 as the only inflammatory marker in the present study.

In conclusion, we showed that elevated TAF IL-6 levels at birth are strongly associated with funisitis. The TAF IL-6 concentration is a useful marker for detecting fetal inflammation in ventilated ELGAN.

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