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# Low Levels of Tissue Inhibitors of Metalloproteinases With a High Matrix Metalloproteinase-9/Tissue Inhibitor of Metalloproteinase-1 Ratio Are Present in Tracheal Aspirate Fluids of Infants Who Develop Chronic Lung Disease

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**ABSTRACT.** *Objective.* The pathogenesis of chronic lung disease (CLD) involves inflammation with proteolytic damage to lung extracellular matrix. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that, acting in concert with their tissue inhibitors, tightly orchestrate extracellular matrix morphogenesis and repair after injury. Imbalances in their levels relative to that of their inhibitors have been implicated in diseases characterized by matrix disruption and remodeling. We investigated the possibility that imbalances in MMP-9 and MMP-2 relative to their tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2, respectively, in tracheal aspirates of preterm infants may be involved in the development of CLD.

*Methods.* Serial tracheal aspirates collected from birth until extubation in 49 ventilated preterm infants (24-32 weeks' gestations) were analyzed for MMP-2, MMP-9, TIMP-1, and TIMP-2. Data normalized by TA values of free secretory component of immunoglobulin A were compared for CLD ( $n = 22$ ) versus no CLD ( $n = 27$ ). Also, known clinical predictors of CLD (gestational age, birth weight, and sex) were assessed for both groups. Association of predictors with the outcome CLD was assessed by logistic regression.

*Results.* Mean gestational age was lower in CLD infants, but birth weight and gender were comparable for both groups. CLD infants had significantly lower TIMP-1 level with higher MMP-9/TIMP-1 ratio during the first 2 weeks of life and low TIMP-2 and MMP-2 levels during the first 3 days of life compared with no-CLD infants. Logistic regression analysis indicated that the findings are predictive of CLD.

*Conclusions.* We conclude that low tracheal aspirate levels of TIMPs, with a high MMP-9/TIMP-1 ratio early in life, are associated with subsequent development of CLD. *Pediatrics* 2004;113:1709-1714; *MMPs, TIMPs, chronic lung disease.*

ABBREVIATIONS. CLD, chronic lung disease; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; RDS, respiratory distress syndrome; TAF, tracheal aspirate fluid; EIA, enzyme immunoassay.

Chronic lung disease (CLD) remains the most prevalent chronic morbidity afflicting the prematurely born infant. Although advances in therapy have improved survival rates for extremely low birth weight infants, an unfortunate consequence has been the rising incidence of CLD with its serious morbidity implications.<sup>1</sup>

The mechanisms underlying pathogenesis of CLD are not fully understood, but proteolytic injury to the lungs, from the actions of enzymes released by an influx of activated inflammatory cells, is a major contributing early event.<sup>2-4</sup> There is evidence that the proteolytic injury is facilitated through triggering of an imbalance in lung proteinase versus antiproteinase defense.<sup>3,4</sup> Studies of this concept to date have focused almost exclusively on the levels of serine proteases, such as elastase, relative to those of their inhibitors  $\alpha$ -1-antitrypsin and secretory leukocyte inhibitor.<sup>4,5</sup> Therapeutic and prophylactic intervention strategies for CLD derived from these studies have yielded disappointing results in trials.<sup>6</sup>

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases with crucial roles in extracellular matrix remodeling as occurs during lung morphogenesis, growth, and repair after injury.<sup>7-9</sup> They are involved in angiogenesis, cell migration, tissue inflammation, degradation, and wound healing.<sup>7-9</sup> MMPs proteolytically cleave extracellular matrix components, with some members demonstrating near-total substrate specificity in their actions. MMPs are secreted as zymogens (pro-MMPs) that require proteolysis for activation, and their activities are tightly regulated by specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs) that bind to active MMPs in a 1:1 stoichiometric ratio.<sup>7-9</sup>

MMP-2 (72-kDa gelatinase or gelatinase A) is secreted mainly by noninflammatory cells (fibroblasts and endothelial and epithelial cells), whereas MMP-9 (92-kDa gelatinase or gelatinase B) is secreted by inflammatory cells (neutrophils, monocyte-macrophages).<sup>7-9</sup> TIMP-1 is the specific inhibitor of MMP-9

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but has overlapping inhibitory effects on other MMPs, including MMP-2. TIMP-2, however, is the specific inhibitor for MMP-2. The production of MMPs is under transcriptional modulation by several factors, including cytokines, growth factors, and extracellular matrix components.<sup>7-9</sup>

Imbalance between MMPs and their TIMPs have been implicated in the pathologic tissue degradation and remodeling that occurs in diseases such as asthma, acute respiratory distress syndrome (RDS), pulmonary fibrosis, and emphysema.<sup>10-13</sup> However, characterization of MMPs and their TIMPs beyond the first few postnatal days of life in the tracheal aspirate of ventilated preterm infants with evolving CLD has not been reported. The current study was undertaken to investigate the concentrations of MMPs and TIMPs in tracheal aspirates of ventilated preterm infants and the possibility that imbalances in their relative levels may be involved in the development of CLD.

## METHODS

### Patients

The study was prospective with enrollment of infants who were <32 weeks' gestation, had birth weight of <1500 g, required mechanical ventilation for respiratory failure, and were admitted into the neonatal intensive care unit at Children's Hospital of Philadelphia and the Children's Mercy Hospital in Kansas City. The infants underwent serial tracheal aspirate fluid (TAF) collections according to standardized protocols. Infants with proven sepsis as evidenced by a positive blood culture, perinatal asphyxia, or major congenital anomalies were excluded from the study. All infants in the study received prenatal steroids for lung maturation, antibiotics for presumed sepsis, surfactant for RDS, and indomethacin for patent ductus arteriosus closure. Gestational age was derived from a combination of pregnancy duration from last menstrual period and physical examination using modified Ballard assessment. Infants who were still dependent on supplemental oxygen to maintain satisfactory oxygen saturation beyond 36 weeks' completed gestation were given a diagnosis of CLD. Forty-nine preterm infants were enrolled in the study; 22 subsequently developed CLD, and 27 did not. Seven infants died, 5 with CLD and 2 without CLD. The dead were included in the data analysis according to their CLD status at the time of death. A summary of the patient characteristics is presented in Table 1. The study was approved by the Institutional Review Boards of both institutions, and informed consent was obtained as recommended.

### Tracheal Aspirate Collection

Beginning on day 0, serial tracheal aspirate samples were obtained at time of routine tracheal suctioning on days 0 to 3, 4 to 10, 11 to 17, 18 to 24, and 25 to 31 of life or until extubation, according to a previously reported method but with minor modifications.<sup>14</sup> Briefly, with infant in the supine position and head brought to midline, 1.0 mL of sterile isotonic saline solution was instilled into the endotracheal tube, followed by 3 to 4 manual breaths. Thereafter, suction was applied at <80 cm H<sub>2</sub>O and the effluent was collected in a Leukens trap. The samples were centrifuged at 500

× g for 15 minutes to remove the cells and the surfactant fraction, with the remaining supernatant stored at -70°C until analyzed.

### Measurement of TIMP-1 in Tracheal Aspirate

TIMP-1 levels in the tracheal aspirates was measured using a commercially available enzyme immunoassay (EIA) kit (Amersham, Piscataway, NJ) according to manufacturer's protocol. A total of 100 mL of serial dilutions of standards and samples was incubated in a 96-well microtiter plate, precoated with monoclonal anti-TIMP-1 antibody for 2 hours at 20°C to 25°C. After washing 4 times, 100 μL of peroxidase-labeled anti-TIMP-1 antibody was added to the wells, followed by additional incubation for 2 hours at 20°C to 25°C. The plate was then aspirated and washed 4 times, followed by detection of bound antibody by incubation with tetramethyl benzidine substrate. The reaction was quenched after 30 minutes at 20°C to 25°C by addition of 100 μL of 1 M sulfuric acid. The optical density was read at 450 nm using a microplate reader. TIMP-1 concentration in the samples was determined by interpolation from the standard curve. Assays were run in duplicate, and the results were averaged. The assay sensitivity level was 1.25 ng/mL, and the interassay coefficient of variation was 13.6%.

### Measurement of TIMP-2 in Tracheal Aspirate

TIMP-2 was measured using a commercially available "double sandwich" EIA kit, Biotrak (Amersham), per the manufacturer's protocol. A total of 100 mL of peroxidase-conjugated secondary antibody to TIMP-2, assay buffer, and standard or sample were mixed in a tube, then 100 μL of the mixture was added to a 96-well microtiter plate precoated with monoclonal anti-TIMP-2 antibody and incubated for 2 hours at 20°C to 27°C. Next, the plate was aspirated and washed 4 times, followed by additional incubation for 30 minutes after addition of tetra-methyl benzidine substrate. The reaction was quenched with 100 μL of 1 M sulfuric acid, and the color was read at 450 nm in a plate reader. The concentration of TIMP-2 in the samples was determined by interpolation from the standard curve. Assays were performed in duplicate, and the results were averaged. The assay sensitivity was 3.0 ng/mL, and the interassay coefficient of variations was 4.8%.

### Assay of MMP-2 and MMP-9

MMP-2 and MMP-9 were measured with a commercially available EIA kit, Biotrak (Amersham). The kits are designed to measure total, active, or free MMP-2 and MMP-9, respectively, in biological fluids. A total of 100 mL of samples and standards per well of a microtiter plate was assayed according to the manufacturer's protocol in duplicate, and the results were averaged. The assay sensitivity for MMP-2 and MMP-9 are 0.19 and 0.125 ng/mL, respectively. The interassay coefficient of variations were 17.9% and 20.9% for MMP-2 and MMP-9, respectively.

### EIA Soluble Secretory Component of Immunoglobulin A

The concentration of free secretory component of immunoglobulin A (IgA) in the tracheal aspirate samples was assayed using a method established in our laboratory.<sup>15</sup> The values obtained were used to normalize that of the MMPs and TIMPs in the samples as previously reported,<sup>16</sup> because, unlike albumin, free secretory component of IgA levels in tracheal aspirate are unaffected by increases in lung capillary leak.

### Albumin Measurement

Albumin was assayed in the samples using a commercially available kit according to the manufacturer's protocol (BCA kit; Sigma, St. Louis, MO).

### Statistics

Data from the TAF measurements were grouped according to postnatal age at time of collection into days 0 to 3, 4 to 10, 11 to 17, 18 to 24, and 25 to 31 for analysis. Patient characteristic data include gender, birth weight, gestation, surfactant, steroids, indomethacin use, and number of deaths. Categorical data were expressed as percentages and continuous data as mean ± standard deviation unless noted otherwise.

Characteristics of the CLD and the no-CLD groups were compared using  $\chi^2$ , Fisher exact, *t*, or Mann Whitney *U* tests as

TABLE 1. Patients' Characteristics

Characteristic	CLD (n = 22)	no-CLD (n = 27)	P Value (Exact)
Birth weight, g	829 ± 265	910 ± 263	.29
Gestation, wk	26.6 ± 2.0	27.9 ± 1.8	.02
Male/female	12/10	9/18	.15
Surfactant therapy	100%	100%	
Prenatal steroids	100%	100%	
Prophylactic indomethacin	100%	100%	
Died	5	2	

appropriate. Bivariate and multivariate logistic regression analysis was used to assess the effect of the predictors TIMP-1 and TIMP-2 and their ratios with MMPs as well as gestational age, birth weight, and gender at the selected time points, on the risk for developing CLD, the outcome variable. Data on TIMPs and MMPs did not seem to be normally distributed but followed a more normal distribution when transformed to log-2 values. Each level was expressed in a box plot as the median, 25th to 75th percentiles, and 10th to 90th percentiles. We used Pearson linear correlation on normalized data to assess the relationship between variables at the time points.  $P < .05$  was considered significant for all statistical tests.

## RESULTS

### Infant Characteristics

A total of 220 tracheal aspirate samples were obtained from 49 infants in the study. Twenty-two infants developed CLD (CLD group), and 27 infants did not (no-CLD group). There were 7 deaths, 5 in the CLD group and 2 in the no-CLD group. The number of serially sampled infants declined with extubation over time to 41 at days 4 to 10; 18 with CLD versus 23 without; and to 22 at days 11 to 17; 12 with and 10 without CLD. The clinical characteristics of infants in both groups were similar, except for mean gestational age, which was significantly lower in the CLD group (Table 1).

### Characterization of MMPs and TIMPs in TAF

MMP-2 and MMP-9, as well as their specific inhibitors TIMP-2 and TIMP-1, respectively, were detected in all of the tracheal aspirate samples. Serial measurements from the same patients showed great variability for levels of MMP-2 and MMP-9, as well as TIMP-1 and TIMP-2. TIMP-1 protein had the highest concentration with levels severalfold that of TIMP-2 in the same sample (Fig 1). Also, TIMP-1 was present at concentrations significantly higher than that of MMP-9, but MMP-2 versus TIMP-2 levels were not different. The ratio of MMP-9 to its specific inhibitor TIMP-1 was 10- to 50-fold less than that of MMP-2 to TIMP-2 in individual TAF samples.

### TIMP-1 and MMP-9 in CLD Versus No-CLD

TIMP-1 concentration was significantly lower in infants who developed CLD versus those who did

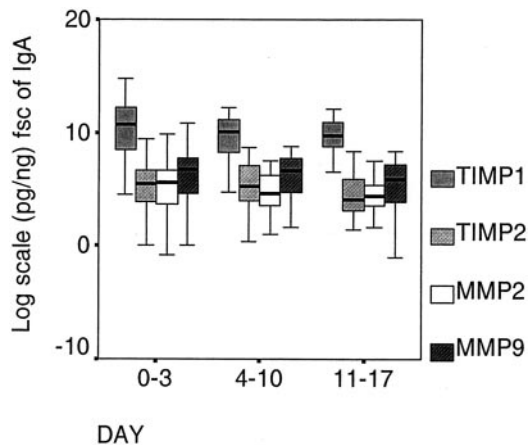


Fig. 1. Graph showing relative levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in TAF samples of all of the infants on days 0 to 3, 4 to 10, and 11 to 17 without regard to CLD status. Note that TIMP-1 concentration is the highest. MMP-2 versus TIMP-2 levels were not different.

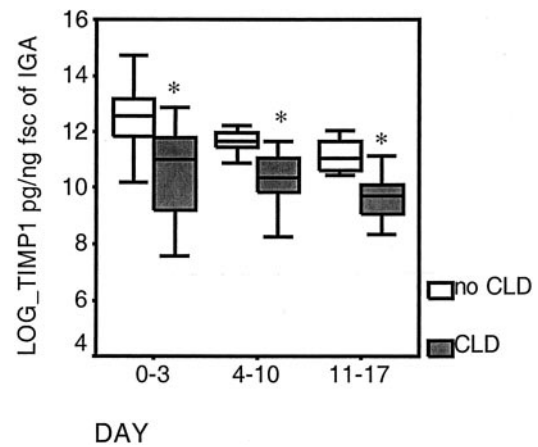


Fig. 2. TAF concentrations of TIMP-1 during the first 2 weeks of life in CLD versus no-CLD infants. Infants who developed CLD had significantly lower levels of TIMP-1 at days 0 to 3, 4 to 10, and 11 to 17 compared with those who did not.  $*P < .05$  CLD versus no-CLD.

not when compared at days 0 to 3, 4 to 10, and 11 to 17 of life ( $P < .05$ ; Fig 2). The ratio MMP-9/TIMP-1 was significantly greater in infants who developed CLD than in those who did not when compared at these same time points ( $P < .05$ ; Fig 3). No significant difference was detected at days 18 to 24 and 25 to 31, respectively, but this may be attributable to sample size limitations as fewer infants remained intubated at these later time points. MMP-9 levels did not differ in the CLD versus no-CLD groups at any of the time points studied.

### TIMP-2 and MMP-2 in CLD Versus No-CLD

TIMP-2 and MMP-2 levels at baseline (days 0-3) were significantly lower in infants who developed CLD versus those who did not develop CLD ( $P < .05$ ; Figs 4 and 5). However, there was no significant difference in the ratio MMP-2/TIMP-2 between these clinical groups. Also, differences in MMP-2 and TIMP-2 levels were not detected at later time points.

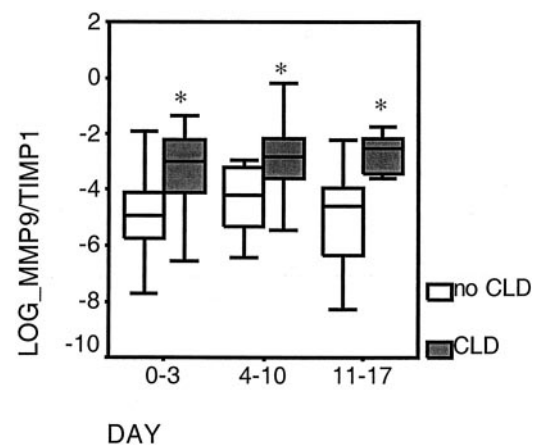


Fig. 3. TAF MMP-9/TIMP-1 ratio during the first 2 weeks of life in infants who developed CLD versus no-CLD. Note the significantly higher MMP-9/TIMP-1 ratios in the CLD group at days 0 to 3, 4 to 10, and 11 to 17 relative to the no-CLD group.  $*P < .05$  CLD versus no-CLD group.

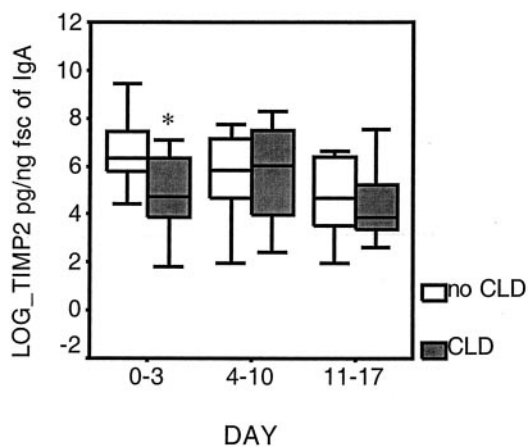


Fig. 4. TAF TIMP-2 levels on days 0 to 3, 4 to 10, and 11 to 17 in infants who developed CLD versus no-CLD infants. Note the significantly lower TIMP-2 level at days 0 to 3 in infants who developed CLD compared with the no-CLD infants. \* $P < .05$ .

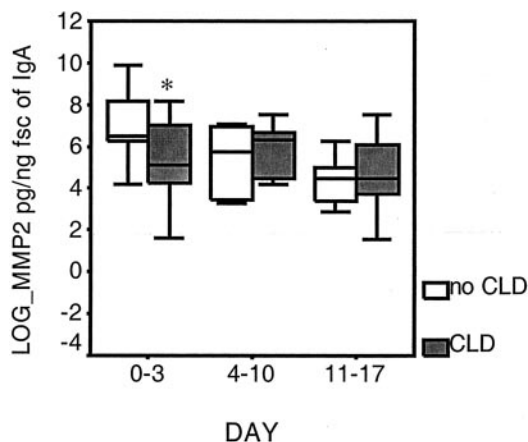


Fig. 5. TAF MMP-2 levels on days 0 to 3, 4 to 10, and 11 to 17 in infants who developed CLD versus no-CLD infants. Note the significantly lower MMP-2 level at days 0 to 3 in infants who developed CLD compared with the no-CLD infants. \* $P < .05$ .

#### Association Between MMPs and TIMPs and Their Ratios and Development of CLD

For determining whether the levels of TIMPs and MMPs or their ratios at the time points correctly predicted CLD status at 36 weeks' postconceptional age, a logistic regression model with CLD as the outcome was developed. Predictors were TIMP-1 and TIMP-2 and their ratios with MMPs as well as gestational age, birth weight, and gender at days 0 to 3, 4 to 10, and 11 to 17. The odds of developing CLD at 36 weeks' postconceptional age was first assessed in univariate logistic models using each predictor separately at the different time points. The results are summarized in Table 2. There was a significant and inverse association between TIMP-1 at days 0 to 3, 4 to 10, and 11 to 17 and the risk for CLD, whereas for the MMP-9/TIMP-1 ratio, the association was direct at these same time points. In contrast, TIMP-2 and MMP-2 levels showed only an association with risk for CLD at days 0 to 3. The risk for CLD was significantly associated with lower gestational age but not with birth weight and gender. When all of the predictors were tested jointly for a combined logistic

TABLE 2. Association Between Predictor Variables and the Outcome CLD at 36 Weeks' Corrected Age as Determined by Logistic Regression

Predictor Variable	Days	Odds Ratio (95% Confidence Interval)	<i>P</i> Value
LG_TIMP-1	0-3	0.42 (0.24-0.75)	.003
	4-10	0.03 (0.001-0.637)	.025
	11-17	0.27 (0.08-0.92)	.036
LG_MMP9/TIMP-1 Ratio	0-3	2.16 (1.26-3.68)	.005
	4-10	2.23 (1.01-4.92)	.047
	11-17	1.87 (1.0-3.53)	.054
LG_TIMP-2	0-3	0.38 (0.19-0.74)	.005
	4-10	1.001 (0.630-1.59)	.998
	11-17	1.037 (0.66-1.64)	.876
LG_MMP-2	0-3	0.55 (0.35-0.88)	.012
	4-10	1.06 (0.63-1.76)	.835
	11-17	1.29 (0.74-2.25)	.375

Data were transformed to log 2 values for variables.

model that best predict CLD, TIMP-1 remained an important factor. Addition of the other predictors to the model did not improve ability to predict CLD beyond that using TIMP-1 alone except at days 0 to 3, when TIMP-2 level was a better predictor.

#### DISCUSSION

In this work, we have characterized the presence and defined the relationships between MMP-2 and MMP-9 and their specific tissue inhibitors TIMP-2 and TIMP-1, respectively, in tracheal aspirates of ventilated preterm infants with evolving CLD. We found low levels of TIMP-1 with high MMP-9/TIMP-1 ratios during the first 2 weeks of life and low levels of TIMP-2 and MMP-2 during the first few days of life in TAFs of infants at risk for CLD. These markers reliably predict CLD at 36 weeks' postconceptional age.

The findings are of clinical importance because they suggest that imbalance in the protease antiprotease system constituted by MMP-9 and its specific tissue inhibitor TIMP-1 may have a role in the pathogenesis of CLD. This has important clinical implications as it raises the prospect of therapeutic and/or prophylactic intervention with anti-MMP agents or TIMP supplementation for CLD.

Previous investigators, including us, have reported data describing the presence of MMPs and TIMPs in TAFs from ventilated preterm infants during the first few days of life.<sup>16-20</sup> However, this is the first report characterizing MMP-2, MMP-9, TIMP-1, and TIMP-2, as well as the relationships among these variables, from birth until extubated or development of CLD. Our finding of no difference in TAF MMP-9 level in CLD versus no-CLD infants is consistent with that of Schock and Sweet et al in their recent study but differs from that of Sweet et al in a previous work.<sup>17,20</sup>

We found lower levels of TIMP-2 and MMP-2 but similar MMP-2/TIMP-2 ratios in TAF samples obtained during the first 3 days of life from infants who later developed CLD compared with those who did not. The observation of low TIMP-2 levels is consistent with that of Cederqvist et al.<sup>16</sup> In their study of TAF samples obtained within the first 5 postnatal

days in infants with RDS, they reported low TIMP-2 levels in those who either had a poor respiratory outcome or later received a diagnosis of bronchopulmonary dysplasia. Danan et al<sup>19</sup> in their study of preterm infants reported a link between low MMP-2 levels at birth and development of CLD. They did not assess TIMP-2 levels and did not detect any association between MMP-9 and TIMP-1 level with development of CLD. Although methodologic differences preclude direct comparison, our findings for MMP-2 and MMP-9 are consistent with their observation but differ with respect to TIMP-1. Our finding of similarity in MMP-2/TIMP-2 ratio of the CLD and no-CLD groups despite lower MMP-2 and TIMP-2 levels in the CLD infants indicates that there was a proportionate decline in the protease and its inhibitor. This finding contrasts with that observed for MMP-9 and TIMP-1, whereby the difference in MMP-9/TIMP-1 ratio between the groups seems to be a consequence of the decreased levels of TIMP-1 in CLD infants. One possible explanation is the absolute dependence of MMP-2 and TIMP-2 production on transcriptional regulation, thus allowing for changes in the levels of both proteins to be tightly controlled. The production of MMP-9 and TIMP-1 is also under transcriptional modulation. However, MMP-9 also exists preformed in neutrophil storage granules that can be released in response to inflammatory stimuli, which would make maintenance of the MMP-9/TIMP-1 ratio a more challenging task.

Extracellular matrix turnover is dependent on the net balance between synthesis and degradation of its constituent proteins such that increased degradation would be expected if the level of proteases rises relative to that of antiproteases, and vice versa. Indeed a high MMP-9/TIMP-1 ratio and low TIMP-1 level have been implicated in the pathogenesis of several pulmonary disorders characterized by extracellular matrix degradation and extensive tissue remodeling, whereas the reverse situation is associated with lung repair and fibrosis.<sup>10-13</sup> Increased elastolysis and elevated levels of extracellular matrix constituent proteins including laminin, fibronectin, and elastin breakdown fragments have also been reported in various biological fluids obtained from infants who developed bronchopulmonary dysplasia.<sup>21-23</sup> These findings have been attributed to imbalance between elastase and its inhibitors  $\alpha$  1 antitrypsin and secretory leukocyte inhibitor.<sup>24</sup> However, the current study has shown, for the first time, that infants who develop CLD have a higher MMP-9/TIMP-1 ratio and lower TIMP-1 levels in their TAFs compared with those who do not.

MMPs and their TIMPs have a recognized physiologic role in orchestration of tissue morphogenesis but have also been implicated in pathologic roles in a growing list of disorders that now seems to include CLD. This has important clinical implications because it presents the possibility for therapeutic/prophylactic intervention for CLD using MMP inhibitors or by supplementation of the tissue inhibitor. This approach has demonstrated encouraging results in experimental models of bronchial asthma and lung injury.<sup>25</sup>

There are several limitations of this study that need to be considered. First, although we have data for total MMP-2 and MMP-9 immunoreactivity in all of the samples, we obtained data for MMP-2 and MMP-9 activity in only a limited number of samples because of insufficient sample volumes. The total level as detected by immunoreactivity includes MMP that is fragmented, bound to TIMPs, or still in the proenzyme form and thus does not reflect activity level. However, other studies implicating imbalance in MMP/TIMP ratio as the basis for disease conditions have relied on the total values.

Second, we restricted the study to preterm infants who were treated with endotracheal intubation because of the need to obtain tracheal aspirate samples for analysis. However, infants who were treated with only continuous positive airway pressure for respiratory support are also at risk for developing CLD. It is not known whether conclusions drawn from our data extend to this population of infants.

Next, is the difficulty presented by the lack of a totally reliable reference protein for normalization of TAF measurements to correct for sample variations that stem from the increased capillary leak that is present in inflamed lungs. The free secretory component of IgA has been used to normalize TIMP and MMPs in tracheal aspirate.<sup>16</sup> Although it has its limitations as a reference protein,<sup>26</sup> it has been shown to be superior to albumin in this regard and, therefore, was used in this study.<sup>27</sup> Furthermore, we also compared values obtained when our data were normalized by TAF albumin, and the results and conclusion were the same.

In summary, we have provided data describing the presence of MMP-2, MMP-9, TIMP-1, and TIMP-2 from birth to the development of CLD in TAF of ventilated preterm infants. Our findings indicate that there is a low level of TIMP-1 with a high MMP-9/TIMP-1 ratio in the first 2 weeks of life, in addition to low levels of TIMP-2 and MMP-2 in the first few days of life in TAF of infants who develop CLD. This suggests that insufficient inhibition of MMP-9 as a result of a low level of TIMPs may be a contributory mechanism to development of CLD in preterm infants; of note, elevated MMP occurs within a few days of birth, a finding consistent with the prevailing view that early lung inflammation is involved in the pathogenesis of CLD. The implications are that early postnatal intervention with MMP antagonists or supplementation of TIMPs could be an effective strategy in the treatment and prophylaxis of CLD. Additional studies are warranted to validate this work and to explore the therapeutic and prophylactic prospects for CLD raised by these findings.

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