ERJ Express. Published on December 5, 2013 as doi: 10.1183/09031936.00105613

Pulmonary MMP-9 Activity in Mechanically Ventilated Children with RSV Disease

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Grant: Pediatric Critical Care Scientist Development Program (PCCSDP; NICHD 5K12HD047349) to MK and HL102371 to AG

Conception and design: MK, JPC, EB, AG; Analysis and interpretation: MK, JPC, NP, YL, XX, TZ, RO, WS; Drafting the manuscript for important intellectual content: MK, JPC, NA, EB, AG.

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Abstract.

Hypothesis: RSV infection is a potent stimulus for airway epithelial expression of MMP-9, and MMP-9 activity *in vivo* is a predictor of disease severity in children with RSV-induced respiratory failure (RSV-RF).

Methods: Human airway epithelial cells were infected with RSV A2 strain, and analyzed for MMP-9 and tissue inhibitor of metalloproteinases-1 (TIMP-1, a natural inhibitor of MMP-9) release. In addition, endotracheal samples from children with RSV-RF and controls (non-RSV pneumonia and non-lung disease controls) were analyzed for MMP-9, TIMP-1, human neutrophil elastase (HNE) and myeloperoxidase (MPO) activity.

Results: RSV infection of airway epithelia was sufficient to rapidly induce MMP-9 transcription and protein release. Pulmonary MMP-9 activity peaked at 48 hours in infants with RSV-RF compared to controls. In the RSV group, MMP-9 activity and MMP-9:TIMP-1 ratio imbalance predicted higher oxygen requirement and worse Pediatric Risk of Mortality scores. Highest levels of HNE and MPO were measured in the RSV cohort but unlike MMP-9, these neutrophil markers failed to predict disease severity.

Conclusions: These results support the hypothesis that RSV is a potent stimulus for MMP-9 expression and release from human airway epithelium, and that MMP-9 is an important biomarker of disease severity in mechanically ventilated children with RSV lung infection.

Word Count: 200

Background.

RSV infection is a common cause of respiratory disease in infants, and can be particularly severe in infants who are premature, have chronic lung disease, congenital heart disease or are immunocompromised (1-2). Furthermore, RSV infection is a trigger for acute exacerbations in chronic lung diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (3-4), and is a risk factor for recurrent wheezing in children post infection (5). Despite the substantial morbidity and mortality associated with RSV infection, treatment remains primarily supportive, and antiviral agents have not proved to be an effective therapy for severe RSV disease.

MMP-9 is one of 25 members of the matrix metalloproteinase family, which consists of ubiquitous enzymes that are capable of degrading various extracellular matrix proteins (6). MMPs are tightly regulated, including binding to peptide inhibitors (such as Tissue Inhibitors of Metalloproteinases; TIMPs-1, -2, and -3), and activation that requires proteolytic cleavage and/or oxidation (7). MMP-9 is prominently expressed in the lung, and while it is involved in many normal homeostatic processes, dysregulated MMP-9 activity has been described in a number of diseases, including asthma (8-9), emphysema (10), COPD (11), bronchopulmonary dysplasia (12) and in neutrophil-dominated pediatric lung diseases including acute lung injury (ALI) and CF (13-15). Recent evidence also suggests that MMP-9 may be a key regulator of neutrophil recruitment through generation of chemotactic collagen fragments *in vitro* and *in vivo* (16-17).

Previously we reported high levels of MMP-9 in the respiratory secretions of pediatric patients with ALI caused by a variety of insults including RSV and other viral infections, and an inverse relationship between MMP-9 activity and ventilator-free days (18). In the current study we focused on relationships between RSV infection of the airway and MMP-9 activity *in vitro* and *in*

vivo. RSV was a potent and specific stimulus of MMP-9 expression and release from primary human airway epithelial cells (HAECs), and MMP-9 activity correlated with important clinical outcome measures in RSV infected infants, including oxygen requirement and PRISM scores.

Material and Methods

Enrollment of PICU patients with respiratory failure: This observational human study was performed in the Pediatric Intensive Care Unit (PICU) at The Children's Hospital of Alabama with the local Institutional Review Board approval (Protocol X080724010). Informed consent was obtained from legal guardians of the subject prior to study enrollment. Table 1 provides a summary of demographic and diagnostic information regarding the pediatric subjects with RSV-induced respiratory failure (RSV-RF; n=42) and controls (non-RSV pneumonia; n=29 and non-lung disease controls; n=46).

Endotracheal aspirate collection and processing: Tracheal aspirates were collected from RSV and control subjects at 48 hours of intubation, and at Day 5 for those who remained mechanically ventilated beyond the first 2 days. Samples were collected via endotracheal tube suctioning using an 8 French suction cannula, and centrifuged at 500*g* for 10 minutes to separate cells and mucus (pellet) from supernatant. Separate aliquots were subsequently saved at 4°C for further analysis.

RSV infection of airway cell cultures: All airway cells were obtained from lung transplant recipients following informed and signed consent under an IRB-approved protocol. First or second passage cells were seeded onto permeable supports and grown at an air:liquid interface using previously described methods (19). The RSV strain A2 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in HEp-2 human nasopharyngeal carcinoma cells (CCL-23, ATCC) using a previously described sucrose purification method (20).

HAECs were infected with RSV at several different multiplicity of infection (MOI). RSV titers were determined by plaque assay techniques in HEp2 cells (21) and expressed as Plaque Forming Unit (PFU)/millimeter. For UV-inactivation of RSV, aliquots of RSV stocks were inactivated by exposure to 1800 mJ of radiation in a Stratalinker UV cross-linker (Stratagene). For studies with heat-killed RSV, aliquots of viral stocks were boiled for 45 minutes (22).

Initial experiments included the examination of MMP and TIMP expression with RSV infection of HAECs at the 48-hour time point. To test the hypothesis that duration of infection also results in increased MMP-9 and TIMP expression, mRNA levels were determined at Days 2, 3 and 6-post infection.

Immunoblots and zymography analysis of MMP-9 from HAECs and endotracheal samples: Samples were subjected to electrophoresis through 7.5% SDS-PAGE gel. The membrane was incubated with primary MMP-9 antibody (MAB 911; R/D Systems), followed by incubation with species-specific IgG horseradish peroxidase conjugates (at dilutions of 1:5000) the next day. The immunoblot was then developed using ECL chemiluminescent kits (GE Healthcare, Piscataway, NJ). Zymography analyses of study samples were performed using previously published techniques (23-24).

Measurement of MMP-9, TIMP, HNE and MPO levels: MMP-9 activity was quantified using an established fluorometric assay (R&D Systems #F9M00). TIMP-1 (R&D Systems #DTM100), MPO and HNE concentrations (Calbiochem #475919 and CBA016; respectively) were measured using established sandwich enzyme immunoassay techniques.

Statistical analysis.

Descriptive statistics were computed for each study variable of interest, including means, standard error of the means (SEM), medians, and ranges. Since the distributions of the data for the tracheal aspirate inflammatory markers (active MMP-9, total MMP-9, TIMP-1, MMP-9:TIMP-1, HNE, and MPO), as well as those for the other continuous variables (days of intubation, days of PICU stay, oxygen, PRISM 12, PRISM 24, and age), deviated from a normal distribution, these data were logarithmically transformed using a log10 scale prior to statistical analysis. The log-transformed variables were determined to follow a normal or approximate normal distribution through the use of stem-and-leaf plots, normal probability plots, and the Kolmogorov-Smirnov test. Demographic and diagnostic comparisons between RSV, disease control, and control subjects were performed using analysis of variance for quantitative variables and the chi-square test for gender. Overall comparisons between RSV, disease control, and control subjects were performed using analysis of variance, while comparisons including covariates of interest such as age, gender, and measures of disease severity (PRISM scores and oxygen concentration), were performed using analysis of covariance. The Tukey-Kramer multiple comparisons test was used as the post hoc test of choice. Comparisons between 48 hour and Day 5 measurements of active MMP-9, total MMP-9, TIMP-1, and MMP-9:TIMP-1 were performed using the Wilcoxon signed-rank test due to the small number of subjects that had available data at both time points. Pearson correlation analysis was used to determine correlations between pairs of quantitative variables and Spearman correlation analysis was used to determine correlations between gender and quantitative variables. Simple and multiple linear regression analyses were used to assess the ability of the inflammatory markers to predict the outcome measures (days of intubation and days of PICU stay) as well as measures of disease severity. Where possible, nonparametric statistical analyses (including the Kruskal-Wallis test and Spearman correlation analyses) corresponding to the above analyses were performed, and these analyses yielded results that are similar to those obtained by the parametric analyses described above. Statistical

tests were two-sided and were performed using a 5% significance level (i.e. alpha = 0.05). Statistical analyses were performed using SAS software (version 9.3; SAS Institute, Inc., Cary, NC).

Results.

RSV infection induces robust MMP-9 expression in primary HAECs

MMP-9 mRNA levels were increased in primary HAECs infected with RSV A2 strain compared to controls following 48 hours of infection (Figure 1A). MMP-9 gene expression was specific to RSV infection, as exposure to killed virus (heat killed, UV light treated) and adenovirus failed to stimulate increases in MMP-9 mRNA. Figure 1B shows that MMP-9 mRNA increased steadily with increasing RSV MOI (0.05-10, 48 hour time-point) and with duration of infection (MOI=0.1, Figure 1C). In contrast to MMP-9, TIMP-1 mRNA levels were similar between RSV infected and non-infected airway epithelial cells on day 2 and 6 post-infection [post-infection (PI), Figure 1D].

We next examined MMP-9 protein release in HAECs post-RSV infection. Figure 2 demonstrates extracellular release of MMP-9 protein after RSV infection (48 hours, MOI=0.1), with greater release observed in the apical compartment relative to the basolateral compartment. MMP-9 protein concentrations correlated with the increased in MMP-9 mRNA seen on day 2 PI (Figure 1). In contrast to MMP-9, TIMP-1 protein was undetectable in the RSV infected cell media (data not shown). Transepithelial resistance (TER) was monitored as a surrogate for monolayer integrity, and in the RSV infected cells, transepithelial resistance did not decrease from the time of infection (mean±SEM=453±62 $\Omega.cm^2$) through Day 4 PI (mean±SEM=722±19 $\Omega.cm^2$).

MMP-9 activity and total MMP-9 levels are elevated in infants with RSV-RF compared to controls

Zymography was initially used to estimate MMP-9 activity in the endotracheal aspirates obtained from control and RSV-RF subjects. At 48 hours of intubation, endogenous MMP-9 level was present in lung secretions of RSV-RF subjects and non-infected controls, with more robust activity seen in infants with RSV-RF (Figure 3A). Significantly higher amounts of active MMP-9 were measured in subjects with RSV-RF when compared to both control groups (Figure 3B - ELISA). Increases in active MMP-9 seen in the RSV-RF cohort remained significant ($p<0.001$) after adjustment for differences in age, gender, disease severity (oxygen requirement and PRISM scores) and other clinical outcome measures (duration of intubation and PICU stay) between the RSV-RF and control subjects by multivariate analysis.

To assess the total MMP-9 levels in tracheal aspirates, aminophenylmercuric acetate (APMA), a known chemical activator of pro-MMP-9 was added to selected samples. Complementary samples without the addition of APMA measured the amount of active MMP-9 present in the samples (Figure 3B). The highest total MMP-9 levels were observed in the RSV-RF subjects compared to the control groups, and this increase in total MMP-9 concentration (including both basal activity and inducible pro-enzyme) remained statistically significant after adjustment for age, gender, disease severity and outcome measures ($p<0.001$).

TIMP-1 concentrations are decreased in RSV-RF leading to elevated MMP-9:TIMP-1 activity ratios

TIMP-1 serves as an endogenous inhibitor of MMP-9 which binds to MMP-9 in a 1:1 stoichiometric ratio (7). The lowest concentration of TIMP-1 was measured in tracheal aspirates from RSV-RF subjects compared to controls (Figure 3C), contributing to significantly higher MMP-9:TIMP-1 activity ratios seen in RSV-RF subjects (Figure 3D). Following multivariate analysis to adjust for differences in age, gender and disease severity and outcome between

RSV-RF, non-RSV pneumonia and non-lung disease controls, the elevated MMP-9:TIMP-1 ratios seen in the RSV-RF cohort remained significant (*p<0.001*) at the 48 hour time point.

Elevated MPO and HNE levels in RSV-RF

As surrogates for neutrophil influx into the lung, MPO and HNE concentrations were measured in endotracheal lavage samples of RSV and control subjects. At 48 hours of mechanical ventilation, RSV-RF patients had the highest levels of MPO and HNE of the three groups (Figure 4). In the RSV cohort, endotracheal MPO and HNE levels demonstrated a positive correlation (r=0.4; *p=0.017*), while no correlation was observed between MMP-9 concentration with either HNE or MPO.

MMP-9:TIMP-1 ratios remained elevated with disease progression in RSV-RF

We next analyzed MMP-9 activity and MMP-9:TIMP-1 ratios in the subgroup of RSV-RF subjects and controls who required more than 48 hours of ventilatory support. In the RSV-RF subjects (n=8), MMP-9 activity remained high with disease progression (at 48 hours mean±SEM=170±90 ng/mg vs. Day 5=399±47 ng/mg; *p=0.05*). TIMP-1 levels were decreased at Day 5 of mechanical ventilation leading to persistently elevated MMP-9:TIMP-1 ratios in the RSV-RF subjects (Figure 5). In contrast, MMP-9:TIMP-1 ratios were decreased with time in both the non-RSV pneumonia (n=13) and non-lung disease control groups (n=9).

Higher MMP-9 activity at 48 hours correlated with higher oxygen requirement and worse PRISM scores in RSV-RF subjects

At 48 hours of intubation, active MMP-9 levels had a positive correlation (r=0.74; *p<0.001*) with oxygen requirement in the RSV-RF subjects, which was confirmed with multiple regression analyses, after adjusting for age, gender and disease severity (eg: PRISM scores) and other clinical outcome measures (length of intubation and PICU stay, Table 2). A positive correlation

was also seen between total MMP-9 with PRISM 12 scores at 48 hours of disease onset ($r=0.41$; $p=0.022$) in the RSV-RF subjects, which were confirmed by multiple linear regression analyses after controlling for age, gender, disease severity and outcome measures (Table 2). In contrast to MMP-9 activity, neither MPO nor HNE levels demonstrated significant correlation with oxygen requirement, PRISM scores, duration of mechanical ventilation or PICU stay in the RSV cohort. In the control groups, no clear correlation was observed between the measured biomarkers (active MMP-9, total MMP-9, TIMP-1, MMP-9:TIMP-1 ratios, HNE and MPO) with clinical markers of disease severity at 48 hours (oxygen requirement, PRISM scores), or subsequent clinical outcome measures (duration of mechanical ventilation, length of PICU stay).

Discussion.

Our results highlight a unique mechanism by which RSV initiates MMP-9 dysregulation, stimulating MMP-9 transcription and MMP-9 protein release from infected human airway epithelial cells (independent of other host cell types). MMP-9 release occurs before loss of airway epithelial monolayer integrity, suggesting that RSV-stimulation of MMP-9 production and release from HAECs is an early step in RSV disease pathogenesis prior to overt epithelial toxicity. In patients with RSV infection, we found elevated levels of active MMP-9 compared to control population and an imbalance of MMP-9:TIMP-1 in RSV infected patients at 48 hours of intubation. MMP-9 activity and MMP-9:TIMP-1 ratios continued to increase over the course of mechanical ventilation in the RSV cohort, in contrast to depressed levels seen in the non-RSV pneumonia and non-lung disease control groups. Thus, our data support the notion that endotracheal MMP-9 is a specific marker for RSV disease severity in mechanically ventilated infants with RSV-induced respiratory failure.

Few studies have examined the relationship between RSV infection and MMP-9 induction in the lung. Yeo et al reported increased MMP-9 release from HEp-2 cells with RSV stimulation at 24 hours post infection (25). In animal models, RSV infection caused an increase in MMP-9 transcription and protein release in BALF (26). In a more recent study, RSV infection in an immortalized human bronchial epithelial cell line caused an up-regulation of MMP-9 transcription, but MMP-9 protein was not detected in the cell culture supernatants (27). The authors concluded that airway epithelial cells were not the primary source of MMP-9 following a paramyxovirus infection, and that the increase in MMP-9 protein release observed in the A549 cells and HEp-2 cells were due in part to the tumorogenic properties of the cell lines. In contrast to these studies, we found significant elevation of MMP-9 transcription and protein release in the RSV infected primary HAECs when compared to control uninfected cells. The use of primary HAECs in our studies may provide a more physiologic model for studies of MMP-9 and human

RSV infection relative to studies in immortalized cell lines and in animal models. In this model, the airway cells demonstrate a pseudostratified, polarized phenotype, including ciliated and goblet cells, and tight monolayer integrity as measured by high transepithelial electrical resistance. Control experiments utilizing UV-inactivated RSV (which eliminates viral infectivity without altering the conformation of viral proteins) and heat-killed RSV (which eliminates viral infectivity and changes protein structures) failed to stimulate MMP-9 expression, indicating the need for live replicating RSV virus to induce MMP-9 production. Furthermore, MMP-9 mRNA levels increased steadily with increasing MOI but plateaued at an MOI=1, supporting the notion that viral replication (vs. structural viral proteins alone) were key in stimulating MMP-9 production.

A variety of other cells produce MMP-9 in the lung including tissue macrophages, sequestered neutrophils and fibroblasts (28). In addition to epithelial cells, it has been shown that TIMP-1 can be produced from both interstitial and alveolar macrophages. Therefore, although our study highlights the impact of RSV on airway epithelial release and regulation of MMP-9, it is possible that RSV also impacts the regulation and release of these proteins from other cell types in the lung.

Although our cellular findings demonstrate the transcription of MMP-9 and release of pro-MMP-9 following RSV infection, our human data suggests that a large proportion of the detectable MMP-9 was in the active form. RSV-RF subjects also had lower TIMP-1 levels, resulting in higher MMP-9:TIMP-1 ratios during acute disease and with time on the ventilator. Our group has recently demonstrated that HNE is capable of converting pro-MMP-9 to active enzyme, and can independently degrade TIMP-1 (14). Here, we found high levels of HNE in the RSV lung samples compared to controls (approximately 6-fold and 21-fold higher than non-RSV pneumonia subjects and non-lung disease controls, respectively). Thus, we speculate that HNE

may be in part responsible for the high levels of active MMP-9 and low TIMP-1 levels observed in our RSV subjects. Furthermore, our *in vitro* studies demonstrated absent TIMP-1 protein release following RSV infection of HAECs, initiating conditions that predict high MMP-9 :TIMP-1 ratios *in vivo*.

An imbalance between MMP-9 and TIMP-1 levels has been shown to correlate with airflow obstruction, diminished lung function and duration of ventilatory support (13-15, 29). Furthermore, excessive MMP-9 activity was also shown to contribute to increased mortality in mice following *Francisella tularensis* infection, as MMP-9 knockout mice demonstrated improved survival relative to MMP-9+ littermates with respiratory tularemia (30). To our knowledge, this is the first study to describe an association between high levels of active MMP-9, total MMP-9 and MMP-9:TIMP-1 ratios with disease severity in mechanically ventilated children with RSV-induced respiratory failure. Although HNE and MPO (markers of PMN infiltration) were elevated in the RSV-RF subjects, no correlation was found between these neutrophil-derived enzymes with disease severity. A recent study demonstrated a link between disease severity of viral lower respiratory tract infections with increased expression of MMP-8 and MMP-9 genes in mononuclear cells and granulocytes (31). However, no differences were noted in total MMP-9 concentrations in nasopharyngeal washes in children with severe disease compared to those with mild or moderate disease, and MMP-9:TIMP-1 ratios were also not increased during acute RSV infection. In their study, mild disease was defined as RSV without hypoxia, while moderate and severe disease was defined as need for oxygen, and mechanical ventilation, respectively. Since only total MMP-9 concentration was measured, it remains unclear if active MMP-9 was different between those with mild, moderate or severe RSV disease. Another study demonstrated that nasopharyngeal samples from infants with RSV bronchiolitis contained increased MMP-9 and TIMP-1 concentrations, and that TIMP-1 correlated negatively with oxygen saturations (27). In contrast, we observed that TIMP-1 alone in lower airway samples

had no correlation with disease severity or clinical outcome measures. It is important to note that the study populations (27, 31) were different from our RSV cohort, in that all of our study subjects had respiratory failure necessitating ventilatory support. Furthermore, our studies focused only on lower airway samples from RSV-infected and control subjects, thus examining relationships between RSV and MMP-9 in the target organ of disease.

One potential limitation to our study is the confounding effect of mechanical ventilation, and the previous reports of elevated MMP-9 expression seen in patients with ventilator-induced lung injury (32). In our study, all controls and study subjects received mechanical ventilation, thus controlling for the effect of mechanical ventilation on MMP-9 activity. Furthermore, high levels of MMP-9 have also been reported in other PMN dominated inflammatory lung conditions such as pneumonia and acute respiratory distress syndrome (15, 33). In this study, consistent with these previous reports, MMP-9, HNE and MPO activity were elevated in the non-RSV pneumonia subjects compared to non-lung injury controls. However, in our experience, the highest levels of MMP-9 activity were observed in the RSV-infected patients, and MMP-9 (active MMP-9 in particular) outperformed MPO and HNE as a predictor of disease severity. Another potential limitation to the findings of this study is the differences in age across study groups. Despite the varied patient demographics, we confirmed increased levels of MMP-9 activity in the RSV-RF subjects compared to the control groups by using multiple regression analysis, adjusting for differences in age, gender and clinical disease severity measures. We acknowledge that the relatively small number of control subjects with respiratory failure secondary to other viruses precluded a direct comparison between RSV and other viral pathogens. Others have shown correlation between high RSV titers and disease severity, suggesting that viral burden is the driving force behind the inflammation and deranged pathology observed in RSV infection (34-35). In our studies, viral load was not quantified in the lung samples, therefore preventing direct analysis of RSV titers and the measured inflammatory markers with clinical outcome measures.

Finally, due to the age and disease severity, bronchoalveolar lavages were not consistently performed in our patient population. We instead utilize endotracheal aspirates as the safest method for sample collection in our studies. It is possible that cellularity and enzymatic profile in the alveolar compartment is different relative to that seen in tracheal-derived secretions. In our studies, we employed standardized sampling techniques and adjusted the MMP, TIMP, HNE and MPO levels for the amount of protein present in the sample. The use of endotracheal samples may also have practical implications, as endotracheal aspirates are simple to obtain and thus may improve the portability of our findings.

Together, our findings suggest that dysregulation of MMP-9 activity and disruption of MMP-9:TIMP-1 homeostasis are key factors contributing to inflammation and clinical disease manifestations in the RSV infected lung. Our *in vitro* results in isolated airway epithelial cell monolayers identified RSV infection as a specific stimulus of MMP-9 expression and release independent of non-epithelial cells, and thus provides a rationale for close correlations between MMP-9 and clinical markers of disease (as opposed to other markers of neutrophil influx). These findings are novel, and justify future validation studies in larger patient cohorts, especially to examine MMP-9 imbalance with other previously reported markers of disease severity in RSV infection, such as viral burden, interleukin-4 and -8 gene polymorphisms and MMP-3 (34-37) as this may provide greater prognostic values than each marker individually.

Conclusion.

Our studies identify a direct link between RSV infection and MMP-9 transcription and protein release in human airway epithelial cells. Dysregulated MMP-9 activity, as demonstrated by high levels of active MMP-9 and MMP-9:TIMP-1 imbalance at 48 hours of intubated correlated with higher oxygen requirement and PRISM scores in mechanically ventilated children with RSV-induced respiratory failure in this single center study. Together, the findings support

investigation of RSV and MMP-9 as a mediator of RSV dependent tissue injury, potentially identifying MMP-9 as a novel biomarker and therapeutic target for RSV disease.

Acknowledgements.

We thank Children's of Alabama PICU nurses and respiratory therapists for their assistance with tracheal aspirate collections. We also thank the Cystic Fibrosis Clinical and Translational Core at The Gregory Fleming James Cystic Fibrosis Research Center of UAB for providing us with primary human airway epithelial cells used in the *in vitro* experiments. Finally, the authors thank Dr. Eric Sorscher, M.D. for helpful discussion of the manuscript.

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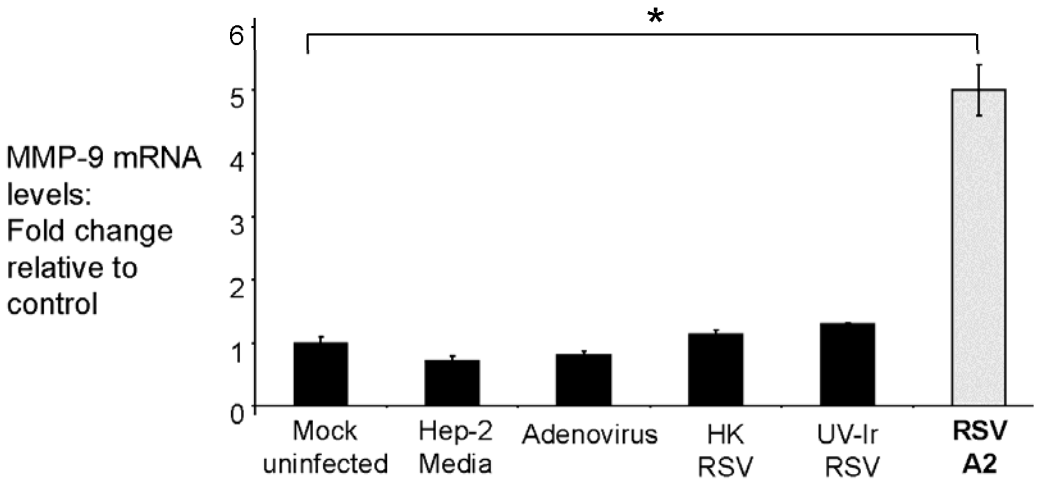
Figure Legends

Figure 1: RSV infection of HAECs resulted in increased MMP-9 transcription, but had no effect on TIMP-1.

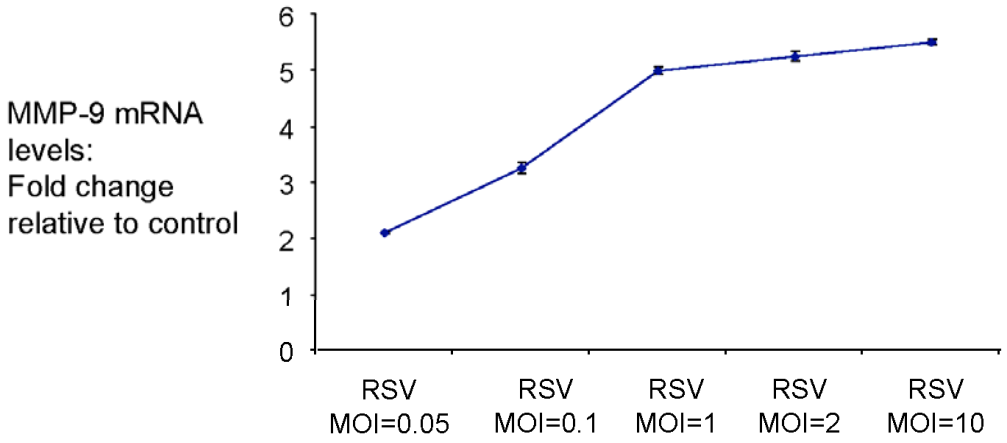
1A: MMP-9 mRNA level was approximately 5-fold higher in human airway epithelia infected with RSV-A2 strain (MOI = 0.1) compared to mock uninfected control at 48 hours post infection (*$p<0.03$). Infection of airway epithelia with replicating adenovirus, heat killed (HK) and UV-irradiated (Ir) RSV resulted in similar levels of MMP-9 transcription when compared to the uninfected cells. Similarly, treatment of cells with Hep-2 media (media used for RSV propagation) had no measureable effects on MMP-9 transcription.

Figure 1A:



1B: Dose/response effects of RSV infection on MMP-9 transcription. Control was MMP-9 mRNA levels measured after infection with RSV at a MOI of 0.01. At day 2 post infection, increasing RSV MOI (0.05-10) resulted in exponential increases in MMP-9 transcription. However, MMP-9 mRNA levels plateaued at a MOI of 1, with similar MMP-9 mRNA levels at MOI of 10 vs. 1.

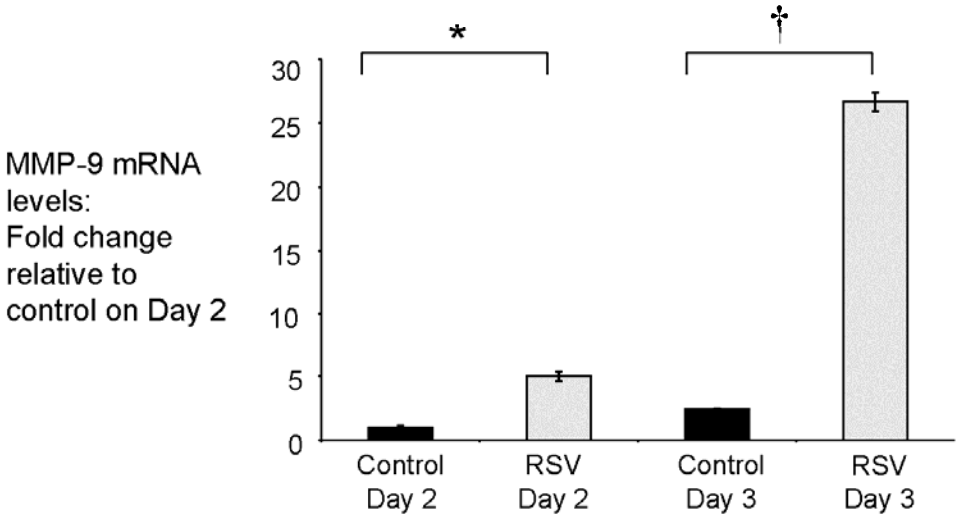
Figure 1B:



1C: MMP-9 mRNA levels in RSV infected cells were 5-fold higher than control (mock-uninfected) at 48 hours post infection (*$p<0.03$), and 11-fold higher compared to non-infected controls at day 3 post infection ($^{\dagger}p<0.002$). Baseline MMP-9 transcription increased with time as seen in non-infected controls (MMP-9 mRNA was 2.5-fold higher in control cells at Day 3

relative to Day 2; *p<0.05*) but the increase in MMP-9 transcription was significantly higher in the RSV infected cells (27-fold increase relative to control cells on Day 2; *p<0.0001*).

Figure 1C:



1D: TIMP-1 transcription was similar between RSV and mock-infected airway epithelial cells on days 2 and 6 post infection.

Figure 1D:

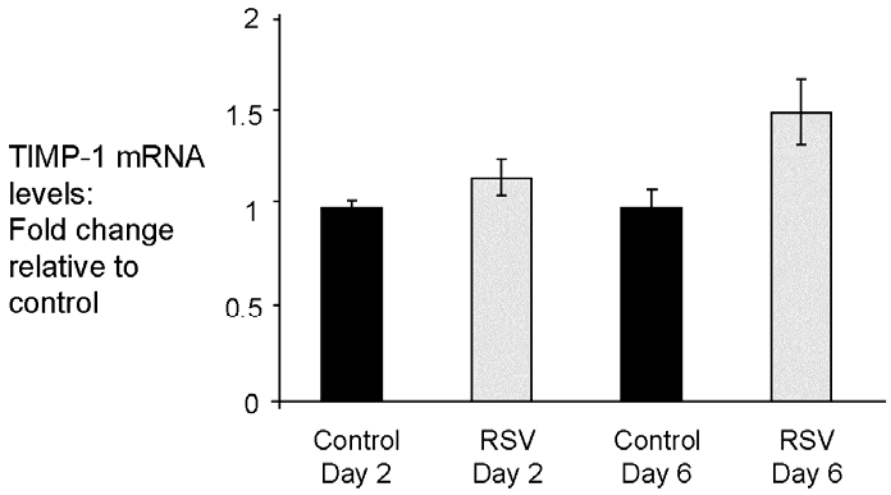


Figure 2: MMP-9 protein release was increased in RSV infected HAECs

MMP-9 protein (92 kDa) was detected in RSV A2 infected cell culture media (at 48 hours) compared to control mock uninfected media. Each lane represents sample from a separate well. 50 μg protein/sample was loaded into each well. *C* represents cell culture media obtained from mock-infected cells (with Hep-2 media) while *R* represents media obtained from RSV-infected human airway epithelial cells (MOI = 0.1). *A* represents 'Apical', which is cell culture media obtained from the apical compartment of RSV-infected monolayers (MOI = 0.1) while *B* represents 'Basolateral' which is media obtained from the basolateral compartment. 50 ng of recombinant MMP-9 (R&D Systems; #WBC018) was used as positive control.

Figure 2:

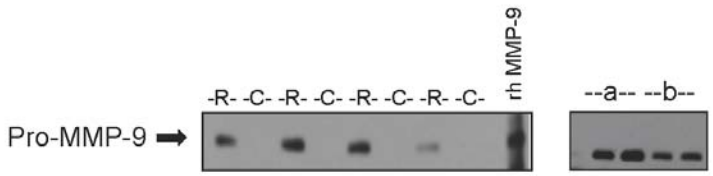
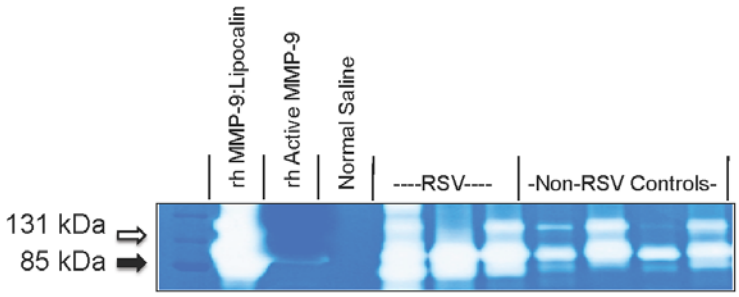


Figure 3: Dysregulated MMP-9 activity was observed in mechanically ventilated infants with RSV-RF

3A: Zymogram (7.5% SDS non-reduced gel) demonstrates robust gelatinase activity in tracheal aspirates from RSV-RF subjects. Each lane represents a sample obtained from separate RSV-RF and control non-infected subjects at 48 hours of mechanical ventilation and onset of respiratory failure. In brief, samples (8 μg protein/sample) were subjected to electrophoresis through 7.5% polyacrylamide gels containing 1 mg/ml porcine skin gelatin in the presence of SDS under non-reducing conditions. Lytic areas within the gel represent MMP-9 activity present in samples. The higher molecular weight band at 135 kDa (white arrow) represents lipocalin:MMP-9 complexes, while the 82 KDa band (black arrow) corresponds with active MMP-

9 isoforms. 25 ng of recombinant lipocalin:MMP-9 complexes (R&D Systems; #444233) and 20 ng of recombinant active MMP-9 (Millipore; #PF024-5UG) was used as positive control. Normal saline (utilized for sample processing) was used as negative control.

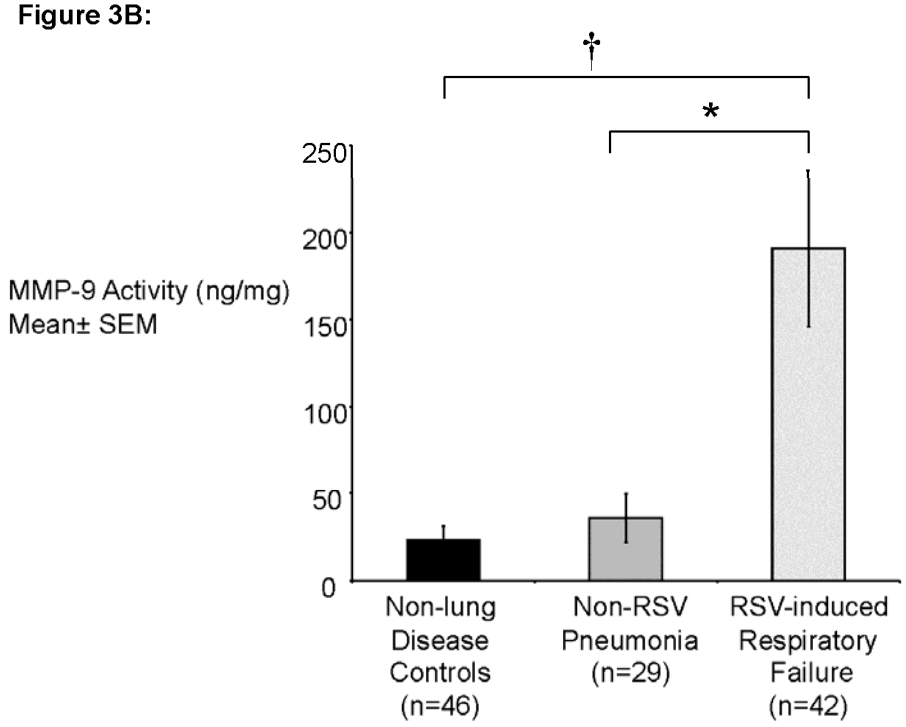
Figure 3A:



3B: The concentrations of endogenously active MMP-9 (i.e. prior to APMA stimulation) were approximately 5 and 9-fold higher in tracheal aspirates from patients with RSV-RF when compared to non-RSV pneumonia and non-lung disease controls, respectively (RSV-RF mean±SEM=190±44 ng/mg vs. non RSV pneumonia mean±SEM=35±14 ng/mg (*$p<0.001$) and non-lung disease controls=22.9±8.1 ng/mg;

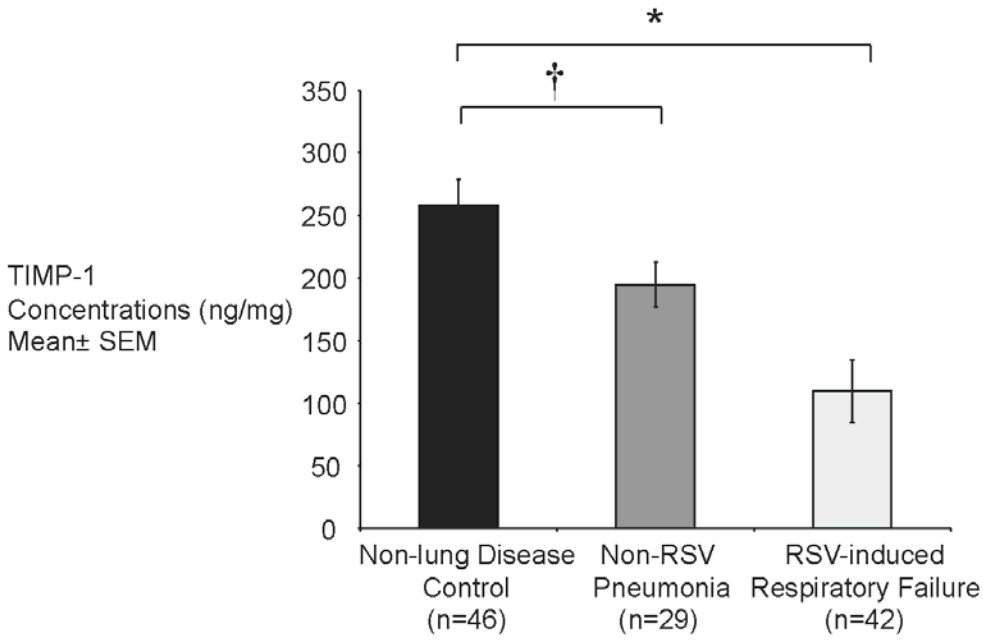
†*p<0.001*).

Figure 3B:



3C: The lowest concentrations of TIMP-1 were measured in the tracheal aspirates from RSV-RF subjects compared to the non-RSV pneumonia and non-lung disease control groups. TIMP-1 level in RSV-RF subjects was approximately 2-fold lower than in the non-lung disease controls (RSV-RF mean±SEM=110±24 ng/mg vs. non-lung disease controls mean±SEM=257±49 ng/mg; **p=0.006*). The amount of measured TIMP-1 in the non-RSV pneumonia group was also lower than the non-lung disease controls (non-RSV pneumonia mean±SEM=194±58 ng/mg; †*p=0.022*).

Figure 3C:



3D: Highest MMP-9:TIMP-1 activity ratios were observed in RSV-RF subjects compared to non-RSV pneumonia and non-lung diease controls (mean±SEM=13.7±2.9 vs. 2.1±0.6 and 2.9±0.6 respectively; *$p=0.005$ and †$p=0.001$), likely secondary to combination of higher MMP-9 activity and depressed TIMP-1 levels found in the RSV subjects (Figure 3B and 3C).

Figure 3D:

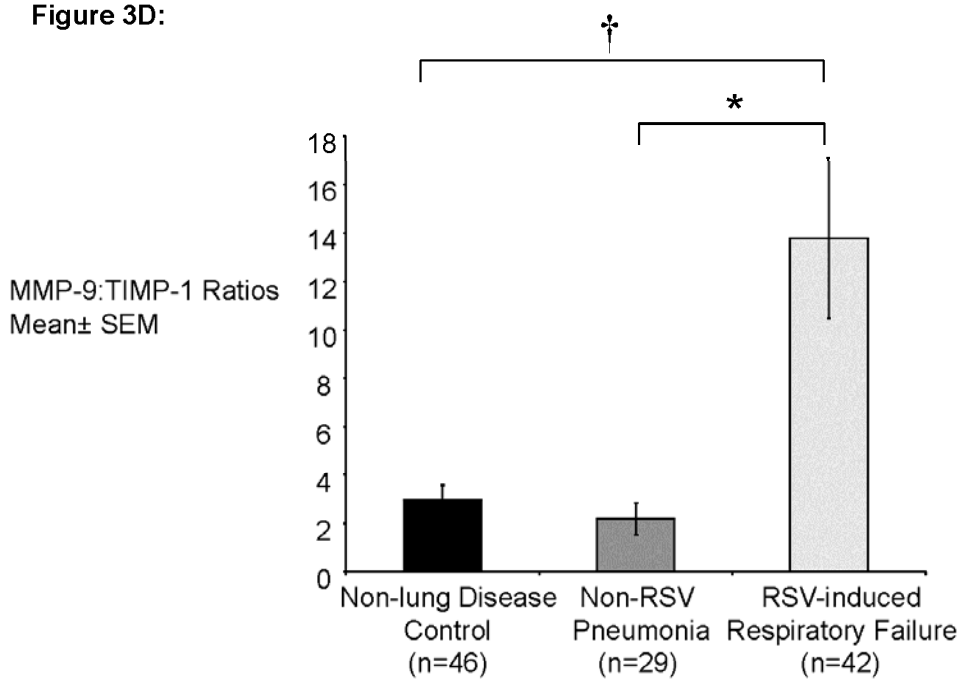
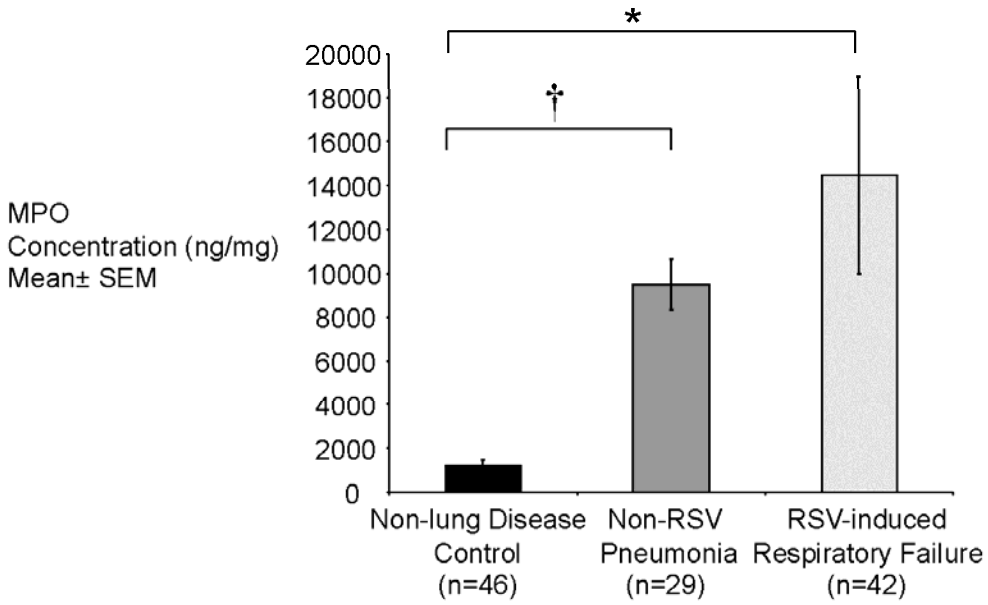


Figure 4: The highest MPO and HNE levels were found in RSV-RF subjects

4A: MPO levels from RSV-RF and non-RSV pneumonia patients were found to be approximately 12-fold and 7-fold higher than in non-lung disease controls (MPO in RSV-RF mean±SEM=14469±4436 ng/mg vs. non-RSV pneumonia mean±SEM= 9486±1154 ng/mg vs. non-lung disease control=1208±278 ng/mg; *$p<0.001$ and †$p=0.001$; respectively).

Figure 4A:



4B: HNE levels were approximately 20-fold and 3.5-fold higher in the RSV-RF cohort and non-RSV pneumonia subjects compared to the non-lung disease controls (HNE in RSV-RF mean±SEM=84±18 ng/mg vs. non-RSV pneumonia controls=14±2.9 ng/mg vs. non-lung disease controls=4.2±0.6 ng/m; *$p<0.0001$ and †$p=0.0001$; respectively). The highest level of HNE was measured in the RSV-RF cohort, with an approximate 6-fold increase seen in this cohort compared to the non-RSV pneumonia controls (‡$p=0.001$).

Figure 4B:

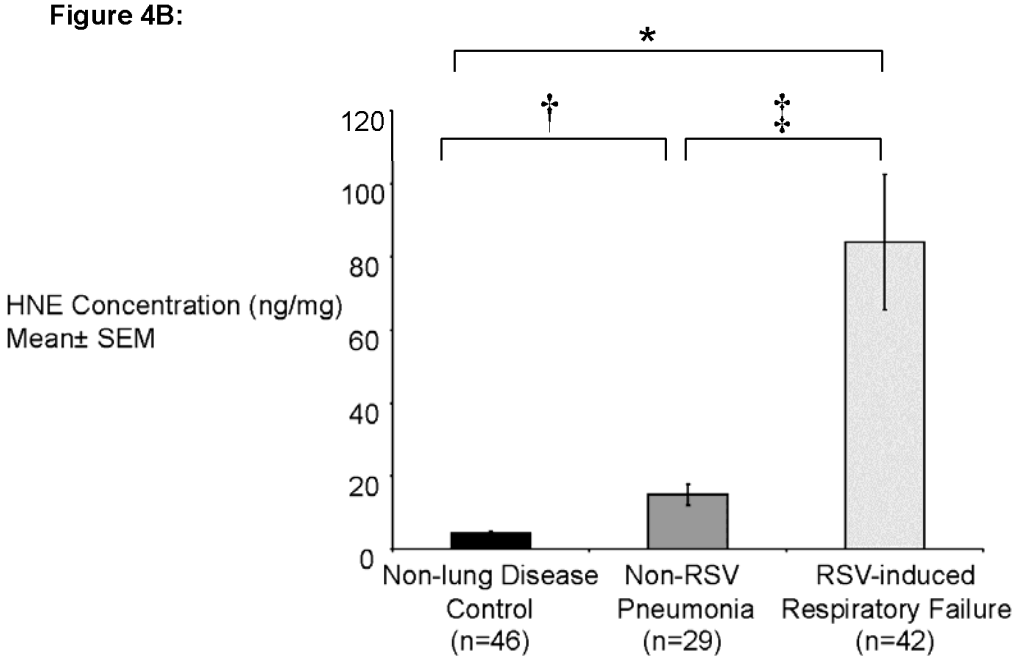
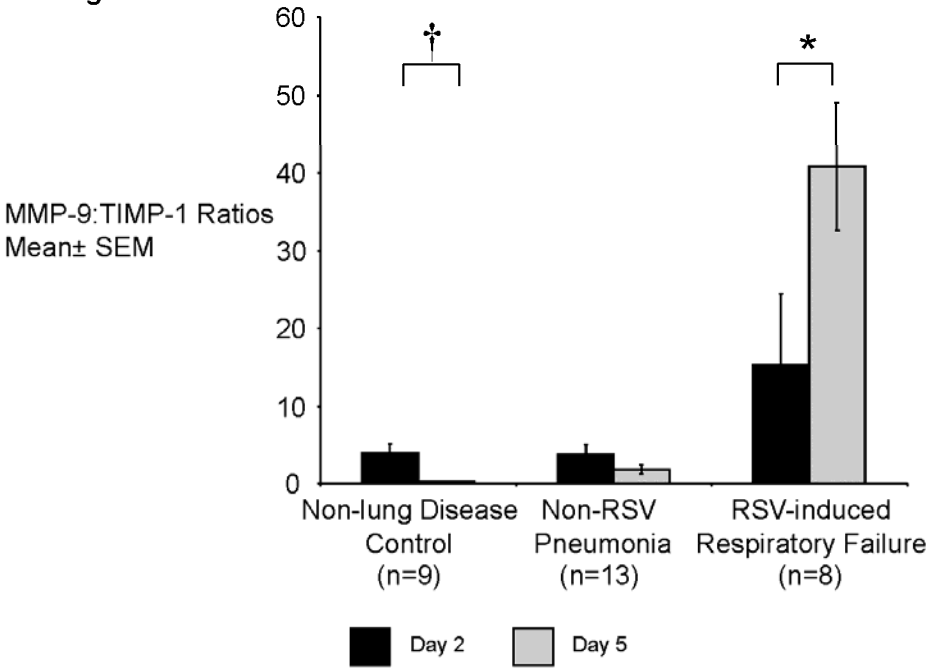


Figure 5: MMP-9:TIMP-1 ratios remained elevated with time in RSV-RF subjects vs. resolution in control groups.

Persistently elevated MMP-9:TIMP-1 ratios were seen with time in the RSV-RF subjects (n=8) during mechanical ventilation (at 48 hours mean±SEM=15±8 vs. Day 5=41±8; *$p=0.04$). In contrast to RSV-RF subjects, non-lung disease controls (n=9) demonstrated lower MMP-9:TIMP-1 ratios in their tracheal aspirates by Day 5 of mechanical ventilation (at 48 hours mean±SEM=4±0.1 vs. Day 5 =0.2±0.05; †$p=0.04$). Similarly, subjects with non-RSV pneumonia (n=13) had a trend towards decreased MMP-9:TIMP-1 ratios (at 48 hours mean±SEM=3.8± 1 ng/mg vs. Day 5 =1.8±0.5; NS) by Day 5 of intubation.

Figure 5:


MMP-9:TIMP-1 Ratios
Mean± SEM
60
50
40
30
20
10
0
†
*
Non-lung Disease Control (n=9)
Non-RSV Pneumonia (n=13)
RSV-induced Respiratory Failure (n=8)
Day 2
Day 5

Tables

Table 1: Demographic and diagnostic information of RSV-induced respiratory failure subjects and controls

	Non-lung disease controls	Non-RSV pneumonia controls	RSV-induced respiratory failure subjects	p
Number	46	29	42	
Age (months): Mean ± SEM (Range)	21.6 ± 4.6 (4 weeks - 11 years)	10.45 ± 2.9 (3 weeks-7 years)	2.8 ± 0.5 (1 week - 5 months)	<0.001
Gender: F:M	14:32	14:15	18:24	0.26
Length of intubation Length of PICU stay Mean days ± SEM	4.9 ± 0.4 6.1 ± 0.4	9.2 ± 1.5 10.03 ± 1.5	5.3 ± 0.6 7.36 ± 0.9	0.006 0.049
PRISM 12 (Mean ± SEM)	4.78 ± 0.8	8.8 ± 1.4	5.7 ± 0.7	0.003
PRISM 24 (Mean ± SEM)	4.47 ± 0.6	6.8 ± 0.8	3.3± 0.6	0.004

Oxygen Concentration (FiO_2; Mean ± SEM)	0.37 ± 0.007	0.63 ± 0.02	0.48 ± 0.02	<0.001
Diagnosis	Closed Head Injury (15) Seizure (9) Post Laryngotracheal Reconstruction (8) Altered mental status (4) Muscular weakness (2) *Others (8)	Bacterial Pneumonia (15) Viral Pneumonia (9) Fungal Pneumonia (2)	†RSV bronchiolitis (32) RSV pneumonia (10)	

* Includes subjects with arrhythmia, posterior spinal fusion, C-spine injuries and nasopharyngeal mass. All the non-lung disease controls had no evidence of acute or chronic lung disease, with minimal ventilator requirements (low FiO_2, positive end expiratory pressure, tidal volume and ventilatory rate) and clear chest radiographs; Bacterial pneumonia includes pneumonia secondary to *Staphylococcus aureus* (n=8), *Pseudomonas aeruginosa* (n=3), *Streptococcus pneumoniae* (3) and *Serratia marcescens* (1) and *Haemophilus influenzae (non-typable)* (1); Viral Pneumonia includes pneumonia secondary to *influenza* (n=5), *adenovirus* (n=3) and *parvovirus* (n=1); fungal pneumonia includes pneumonia secondary to *Candida albicans* (n=2); All control subjects who developed new radiographic changes while on the ventilator or acquired a new viral or bacterial infection were excluded from the study.

†RSV infection was defined as having a positive RSV culture on admission to the hospital.

Table 2: Positive correlation between measured biomarkers in the RSV cohort with clinical measures by multiple linear regression (statistically significant r^2 values reported in table)

	PRISM 12	PRISM 24	Oxygen Concentration	Length of Intubation	Length of PICU Stay
Active MMP-9	NS	NS	0.57(p<0.001)	NS	NS
Total MMP-9	0.41 (p=0.04)	NS	0.37(p<0.001)	NS	NS
MMP-9:TIMP-1 ratio	NS	NS	0.35 (p=0.002)	NS	NS
MPO	NS	NS	NS	NS	NS
HNE	NS	NS	NS	NS	NS

NS=not significant