CISATRACURIUM-INDUCED EFFECTS ON MICROVASCULAR ENDOTHELIAL AND LUNG EPITHELIAL CELLS

by

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(Under the Direction of Somanath P.R. Shenoy and Andrea Sikora Newsome)

ABSTRACT

Acute respiratory distress syndrome (ARDS), a severe condition of acute lung injury (ALI) is associated with hypoxemic lung damage, inflammation, respiratory failure, and high rates of mortality. Matrix metalloproteinase protein-3 (MMP3 or Stromelysin-1) is known to promote vascular injury in ALI/ARDS. Cisatracurium, a nicotinic neuromuscular blocker, is used in ARDS patients to decrease mechanical ventilator dyssynchrony, increase oxygenation, and improve mortality. There is a gap in the knowledge of the potential protective benefits of cisatracurium on the lung endothelial and the epithelial cells and the underlying mechanisms. The objective of the current Master of Science Research dissertation is to investigate the effect of cisatracurium on MMP3 expression/activity in endothelial and lung epithelial cells, in turn, preventing lipopolysaccharide (LPS)-induced damage. In our results, although cisatracurium decreased MMP3 expression/activity and increased expression of cell junction proteins, its effects on cell-cell permeability and pro-inflammatory pathways were modest.

INDEX WORDS: Cisatracurium; MMP3; Stromelysin-1; Lipopolysaccharide; Cell junction

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DEDICATION

This thesis is dedicated to my dear family, my parents Wafeek and Amna, my sisters Raghda and Reem, they have been supportive and give me all the love and care in the world. To my dear Marwa who has always been supporting me at each step, to my dearest friend Raghda, my grandparents Laila and Mohamed El-Hady who left us years ago but their influence still present. Last but not least, I would like to express my sincere thanks to my big family and friends back in Egypt, and to my dear friends here in Augusta. I am blessed to have you all in my life and becoming my support system.

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1. Introduction and Literature review:

1.1. Acute Respiratory Distress Syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) is a hypoxemic inflammatory lung injury that causes respiratory failure. ARDS is responsible for a high mortality rate among critically ill patients with a 35-45% mortality rate in severe forms. The timing of ARDS is the onset of one week of a known clinical insult or new or worsening respiratory symptoms.

In 2012, the consensus for categorization and diagnosing ARDS came out with the Berlin criteria, which were validated in over 4,000 patients based on hypoxemia, the severity of hypoxemia; (ratio of the partial pressure of arterial oxygen to the fraction of inspired oxygen [PaO_2/FiO_2] to; mild (200 mm Hg$<PaO_2/FiO_2 \leq$300 mm Hg), moderate (100 mm Hg$<PaO_2/FiO_2 \leq$200 mm Hg), and severe ($PaO_2/FiO_2 \leq$100 mm Hg). This update for ARDS categorization for diagnosis included the key criteria of ARDS specifying the minimum positive end-expiratory pressure (PEEP) of 5 cm H_2O, (PEEP can increase oxygenation). Further, this update contributed to establishing the minimum standard for mechanical ventilation settings for oxygenation for diagnosing. Importantly, the Berlin definition acknowledges the diagnosis of ARDS in the presence of cardiac failure, diagnosis of ARDS required to have a new respiratory failure or worsening the chronic respiratory disease within 7 days, including chest computed tomography (CT) as an alternative form of imaging as a clinical manifestation for lung

infiltrations, CT imaging with bilateral opacities not fully explained by effusions, lung collapse or nodules. (Thompson, Chambers et al. 2017).

Currently, the lack of early screening methods or biomarkers available for ARDS and late diagnosis increases the rate of mortality. The LUNG SAFE trial was an international, multicenter, prospective study conducted in over 29,000 patients in 50 countries (Bellani, Laffey et al. 2016). The results highlighted that ARDS was not identified by the primary care clinician in almost 40% of cases. In the case of mild ARDS, only 51% of cases were identified and about 78.5% in severe ARDS. When all ARDS criteria were met, only 34% of ARDS patients were identified, suggesting that there was a delay in adapting to the treatment. The ICU admission and hospital mortality ranged from ARDS to 35.3% and 40.0%, respectively.

Despite all the clinical trials that have been done since the 1980s investigating and studying potential pharmacological treatment for ARDS, there is no successful pharmacological treatment to manage or treat ARDS patients. According to the updated guidelines, the current management approaches are limited to supportive care pharmacological and non-pharmacological strategies (Bellani, Laffey et al. 2016).

❖ Standard supportive care modules based on the ARDSnet protocol network and guidelines:

- Sedation and paralysis to decrease the dyssyncronizing on mechanical ventilation and increase oxygenation. The neuromuscular blocking agents (NMBA) used in the guidelines is cisatracurium, but not recommended for routine use.
- Hemodynamic monitoring and nutritional support and conservative fluid management.
- Glucose control, deep vein thrombosis prophylaxis, gastric ulcer prophylaxis.
- Glucocorticoids (methylprednisolone) but not recommended for routine.

(Papazian, Aubron et al. 2019)

❖ Management of hypoxemia:

- Mechanical Ventilator settings that supply High FiO_2, low tidal volume strategy (6 mL/kg), target plateau pressure of 30 cm H_2O or less and permissive hypercapnia.
- Prone positioning is found to improve oxygenation by improving lung perfusion and reduce lung compression. Also, a randomized trial (PROSEVA) suggests a mortality benefit among patients with severe ARDS. (Guerin, Reignier et al. 2013)
- Extracorporeal membrane oxygenation (ECMO) for severe cases.

(Papazian, Aubron et al. 2019)

1.2. Usage of neuromuscular blockers (NMBAs) in the management of ARDS

Mechanically ventilated patients with ARDS suffer from dyssynchrony causing ventilator-induced lung injury (VILI) and increased mortality. Guidelines recommend NMBA to reduce refractory hypoxemia but only recommended for severe ARDS patients with less Pao_2/FiO_2 than 150 mm Hg (Hall 2013) (Murray, DeBlock et al. 2016). Data from the clinical trial were conducted only on cisatracurium, no other NMBAs were studied in ARDS. Also, it's unknown if the administration of cisatracurium for shorter or longer duration could overcome its adverse effect of muscle weakness. (Murray, DeBlock et al. 2016). NMBA improves patient-ventilator synchrony, which has a myriad of effects including improved oxygenation, lower risk of asynchrony-related alveolar collapse, lower incidence of pneumothorax, and reduced VILI (Fanelli, Morita et al. 2016). Recent studies showed patients on NMBA have demonstrated lower pro-inflammatory mediators such as the interleukins. Thus, NMBA administration decreases the inflammatory response associated with ARDS. It was found that cisatracurium, one of the NMBAs is acting through the inhibition of nicotinic acetylcholine receptor-α1 (Fanelli, Morita et al. 2016). Whether cisatracurium has any anti-inflammatory effects outside of the class effect of NMBAs is unclear.

1.3. Pros & cons of using NMBAs and light sedation in ARDS

The pros of using NMBAs include the beneficial effects of improving oxygenation and avoiding ventilator dyssynchronization (Papazian, Aubron et al. 2019). NMBA facilitates the treatment of patients with increased muscle activity like status asthmaticus and prevents unwanted motor movement in respiratory distress (Renew, Ratzlaff et al. 2020). Whereas the ACURASYS (the ARDS et Curarisation Systematique), a clinical trial showed benefits in decreasing the mortality rate of ARDS patients (Papazian, Forel et al. 2010). The ROSE (the Reevaluation of Systemic Early Neuromuscular Blockade) trial in 2019 showed no significant mortality benefit (National Heart, Blood Institute et al. 2019) The cons for NMBAs include prolonged neuromuscular weakness, which can affect the weaning of the patients off the ventilator (Bourenne, Hraiech et al. 2017). The pros for lighter sedation for ARDS patients is that it will avoid the adverse effect of neuromuscular weakness following the NMBAs by decreasing the time on ventilators (Shah, Girard et al. 2017) (Pearson and Patel 2020) and leading to less nosocomial infections. The cons of using sedatives for lighter sedation include the dyssynchronization of patients with severe ARDS on the mechanical ventilators and prevent higher morbidity associated with excessive sedation(Bourenne, Hraiech et al. 2017). The Richmond Agitation-Sedation Scale (RASS) helps to meet the sedation goals effectively.

1.4. Use of NMBAs in ARDS patients.

The ACURASYS and ROSE studies are two large clinical trials conducted to determine the efficiency of NMBAs in ARDS patients (National Heart, Blood Institute et al. 2019). The ACURASYS trial, a multicentered, double-blinded randomized trial that included 340 patients with severe ARDS within the previous 48 hours, was conducted by a group of French researchers between March 2006 to March 2008 at 20 ICU. The patients were randomly assigned to receive

cisatracurium besylate intravenously or placebo. The hazard ratio for death at 90 days in the cisatracurium group, as compared with the placebo group, was 0.68 (95% confidence interval [CI], 0.48 to 0.98; P=0.04), after adjustment for both the baseline PaO_2:FiO_2 and plateau pressure and the Simplified Acute Physiology II score. The crude 90-day mortality was 31.6% (95% CI, 25.2 to 38.8) in the cisatracurium group and 40.7% (95% CI, 33.5 to 48.4) in the placebo group (P=0.08). Mortality at 28 days was23.7% (95% CI, 18.1 to 30.5) with cisatracurium and 33.3% (95% CI, 26.5 to 40.9) with placebo (P=0.05). In the ACURASYS study, the early administration of NMBAs in severe ARDS patients improved the adjusted 90 days of survival and increased the time off the mechanical ventilator without increasing muscle weakness. (Papazian, Forel et al. 2010)

The Prevention and Early Treatment of Acute Lung Injury (PETAL) clinical trial network of the National Heart, Lung, and Blood Institute (NHLBI) conducted the ROSE trial(National Heart, Blood Institute et al. 2019). This was a multi-centered, randomized control, interventional, non-blinded study with one intervention arm with cisatracurium infusion for 48 hours, and the other arm with patients treated with sedatives to achieve light sedation targets. The major goal was to re-evaluate the systemic early use of NMDA in ARDS patients by comparing the effects of both neuromuscular blockade and deep sedation (lighter sedation targets) in the current practice. Because of the concerns of the negative outcomes of the usage of NMBAs in ARDS patients and to determine if early administration would be beneficial the ROSE trial was designed to use lighter sedation doses as compared to the ACURASYS trial (deep sedation was used in both the intervention group and the control group in the ACURASYS trial). The primary endpoint was in-hospital death in 90 days from any cause, which occurred in 213 patients (42.5%) in the intervention group and 216 patients (42.8%) in the control group (between-group difference, −0.3

percentage points; 95% CI, −6.4 to 5.9; P=0.93). Cardiovascular SOFA (Sequential Organ Failure Assessment) scores were higher in the intervention group than in the control group on day 1 (between-group difference, 0.2; 95% CI, 0.1 to 0.4) and day 2 (between-group difference, 0.3; 95% CI, 0.1 to 0.5). However, no differences were observed thereafter, on total SOFA scores or other organ-specific SOFA scores. The study concluded that "among patients with moderate to severe ARDS who were treated with a strategy involving a high PEEP, there is no significant difference in mortality at 90 days between patients who received an early and continuous cisatracurium infusion and those who treated with a usual care approach with lighter sedation targets." Thus, the two clinical trials resulted in the opposite conclusions and recommendations for the benefits of the early usage of systemic neuromuscular blocking agents in ARDS patients regarding the same primary endpoint which is 90 days survival rate. Limitations for the ROSE trial included:

- Lack of blinding may have influenced shorter assessments of neuromuscular function, the level of physical activity, and reporting adverse events.
- They did not systematically measure the effect of neuromuscular blockade on ventilator dyssynchrony. As in patients with ARDS or at risk for ARDS, NMBA eliminates ventilator dyssynchrony effectively.
- Prone positing was done in a smaller number of patients than done in ACURASYS, so it can explain the high mortality rate.
- They included patients who might not have survived long enough.

Despite the conflicting data from the two large clinical trials, the guidelines stated in the light of the ROSE clinical results, it will continue to suggest not to routinely administer an NMBA to patients with ARDS. Further clinical trials will be needed to determine the benefits of NMBAs

in ARDS patients and to further update the guidelines on the use of NMBAs in supportive management of ARDS. (Huang, Angus et al. 2017, National Heart, Blood Institute et al. 2019).

1.5. Pathophysiology of Acute Lung Injury (ALI) and ARDS

The etiologies of ARDS, a severe stage of acute lung injury (ALI) include hypoxia, viral or bacterial pneumonia, inhaling toxins, aspiration of gastric contents, sepsis, and major trauma, etc (Matthay, Zemans et al. 2019). ARDS is associated with diffusive alveolar damage, the congestion of capillaries, intra-alveolar hemorrhage, atelectasis, and alveolar edema, followed by hyaline-membrane formation, epithelial-cell hyperplasia, and interstitial edema days later (Gonzales, Lucas et al. 2015). The clinical course and presentation for ARDS have three sequential phases, namely an exudative, proliferative, and fibrotic phases. The transition from each stage to the next and the deterioration of ARDS happens rapidly.

The exudative phase is known as the first immune response of the lungs to injury and is also known as the acute phase (~7days) of ARDS (Walkey, Summer et al. 2012). This phase is characterized by innate immune cell response causing alveolar epithelial and endothelia barriers disruption leading to the accumulation of plasma, plasma proteins, and cellular content within the interstitium and alveolus causing the flooding of the air space and the interstitium (Blondonnet, Constantin et al. 2016). Classically, ARDS is known to be a neutrophil-driven disease (Potey, Rossi et al. 2019) until the involvement of the innate and adaptative cell immunity in the pathogenies of ARDS is widely known.

The inflammatory response started with the resident alveolar macrophages, their secretion of pro-inflammatory cytokines, recruitment and activation of neutrophils and monocytes, and the activation of alveolar epithelial and effector T-cells (Han and Mallampalli 2015), all causing the propagation of the initial exudative phase insult which sustain the inflammation and tissue injury.

Also, the epithelial injury at that phase causes the dysfunction of the surfactant, with more damage caused to the epithelial cells, which leads to the loss of the surfactant production affecting the alveolar function (Whitsett, Wert et al. 2010). Also, the damage happened to the endothelial cells and the microvascular injury contributing to the air-barrier dysfunction and getting more worse by the mechanical stretch (Herrero, Sanchez et al. 2018)

The combination of epithelial and endothelial lung injury contributes to ventilation/perfusion mismatch [ventilation/perfusion ratio is the efficiency of the matching between the air that reaches the alveoli (ventilation) and the blood the reaches the alveoli via capillaries (perfusion)] and the loss of hypoxic pulmonary vasoconstriction leading to refractory hypoxia (Sweeney and McAuley 2016).

The proliferative phase, that could last for ~3 weeks in survivors is described as the recovery phase that is essential for host survival, with restoring ATII cells (Alveolar cells type II) and the differentiation of into ATI cells (Alveolar cells type I) (Thompson, Chambers et al. 2017). The reestablishment of the epithelial integrity and the barrier permeability function will cause the reabsorption and clearance of the exudative fluid, restoring the alveolar functional architecture (Matthay, Ware et al. 2012). The inflammatory cells play a role in clearing the remaining debris and the vasomotor tone resume to be normal again leading to improving the oxygenation and the slow recovery to the pulmonary compliance (Thompson, Chambers et al. 2017).

The fibrotic Phase is associated with high mortality in patients and is characterized by persistent intra-alveolar flooding, intra-alveolar coagulation, and widespread, extensive extracellular matrix disposition leading to intestinal and intra-alveolar fibrosis (Thompson, Chambers et al. 2017). The extensive fibroblast differentiation to myofibroblasts and the

deposition of alveolar collagen occurs, the failure of its removal leading to the development of cystic changes thus hampering the recovery (Thompson, Chambers et al. 2017).

1.6. Cell junctions and the regulation of vascular permeability

The cell junctions in the lung are responsible for oxygen and carbon dioxide exchange across the distal alveolar-capillary unit, the selective barrier function of the healthy normal lung to solutes and fluids (Matthay, Zemans et al. 2019). The adherens junctions (AJs) and tight junctions (TJs) in both endothelial and epithelial cell layers play a role in the barrier selectivity of lungs. In the alveoli, the alveolar epithelium is lined by two types of cells, flat alveolar type I (ATI) along with cuboidal-shaped alveolar type II (ATII) cells, tother controlling the barrier integrity by permitting only the diffusion of gases and restricting the passage of tiny solutes (Koval 2013). The ATII cells produce the surfactant which is crucial in maintaining the alveoli open for allowing the gas exchange and decrease the surface tension(Whitsett, Wert et al. 2010). When the alveolar edema occurs during ARDS, ATII and ATI are responsible for absorbing the excess of fluids from airspaces by ion transport channels, mainly apical sodium channels and basolateral Na+/K+-ATPase pumps (Matthay, Zemans et al. 2019). Although ATII cells are the precursors of the ATI cells, ATI cells cover most of the alveolar space (> 80%) with ATII cells covering the small surface of the alveolar space (Herzog, Brody et al. 2008). The TJs between ATI cells are responsible for the alveolar barrier function and the regulation of physiological and pathological permeability.

The draining of the edema fluid in the alveoli occurs through the lymphatics and lung microcirculation. The inter-endothelial junctional structure is very similar to that of the lung epithelial (Wallez and Huber 2008). The junctions in the endothelial layers formed by TJ proteins such as the transmembrane claudins and occludins, and intracellular zona occludens that anchor

TJs to the actin cytoskeleton (Zhang and Yang 2016). The claudins in the lung include Claudin 1, 3, 4, 5, 7, 8, and 18 (Wallez and Huber 2008), (Koval 2013). The AJ contains VE-Cadherins (Vascular Endothelial Cadherins) and vascular endothelial- protein tyrosine phosphatase (VE-PTP), which dephosphorylates VE-cadherin and making it stable (Carra, Foglia et al. 2012), thus keeping vascular permeability and leukocyte transmigration (Matthay, Zemans et al. 2019). Studies have shown that VE-cadherin degradation occurs by matrix metalloproteinases, such as the MMP2, 7, and 9 (Rundhaug 2005). While Claudin 18 expressed mainly in the alveolus, collectively Claudins expressions are dysregulated in alcoholic lung disease, sepsis, and pulmonary fibrosis causing lung edema. Studies showed that claudin-5 is highly expressed in the lung endothelial cells while it was barely expressed in the alveolar ATII epithelial cells (Fernandez, Koval et al. 2007). In lipopolysaccharide (LPS)-induced experimental ALI models in-vitro and in-vivo, claudin-5 expression was reduced associating with lung edema (Artham, Gao et al. 2019).

1.7. The structure, function, and role of MMPs in the regulation of vascular permeability

Extracellular Matrix (ECM), is critical for morphogenesis, tissue healing, remodeling, and hemostasis (Nagase, Visse et al. 2006). The ECM is degraded by a class of proteinase enzymes named Matrix Metalloproteinases (MMPs). With their activity as proteolytic enzymes, with a zinc ion (Zn2+) in their catalytic site (Nagase, Visse et al. 2006), (Frantz, Stewart et al. 2010). The MMP family is divided into 6 classes; collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other non-classified MMPs (Jablonska-Trypuc, Matejczyk et al. 2016). There are 23 MMPs in humans and each one has a different role under physiological and pathological conditions (Nagase, Visse et al. 2006). MMPs are secreted as proenzymes. They are secreted in their inactive form called zymogen (pro-MMPs) into the extracellular space, where

they remain inactive until the interaction between catalytic zinc ion and the cysteine residue and the disruption and the proteolytic cleavage at pro-peptide domain occurred (Nagase, Visse et al. 2006). MMPs can be activated by other proteases or by reactive oxygen species (ROS) (Loffek, Schilling et al. 2011). MMPs can be maintained in the inactive stage also by a class of enzymes called the Tissue Inhibitors of Metalloproteinases (TIMPs) (Lech, Wiera et al. 2019). Breaking and disruption of ECM alter cell-cell and cell-matrix interaction leading to the release of growth factors that are bound to the ECM making them available for binding to cell receptors and exert their actions (Bonnans, Chou et al. 2014). Thus, MMPs play a role in cell migration, differentiation, growth, inflammation, neovascularization, morphogenesis, and apoptosis (Nagase, Visse et al. 2006). Junctional proteins are also the targets of MMPs as they are found to cause degradation for VE-Cadherin and Occludin affecting vascular permeability (Alexander and Elrod 2002). E-cadherin degradation by MMP 3 and 7 has been reported in increased cell invasion. (Nagase, Visse et al. 2006)

1.8. Stromelysin1 or Matrix Metalloproteinase3 (MMP3)

MMPs play an important role in innate immunity and inflammatory responses (Greenlee, Werb et al. 2007). Various inflammatory lung diseases such as ARDS, asthma, and pulmonary fibrosis are characterized by an increase in the expression of one or more of the MMPs (Loffek, Schilling et al. 2011). Recent studies in our laboratory have identified the therapeutic benefits of targeting MMP3 (stromelysin1) (Artham, Gao et al. 2019) as well as measuring MMP3 activity in biological fluids such as the plasma and bronchoalveolar lavage (BAL) (Artham, Gao et al. 2019) (Artham, Verma et al. 2020). The exudative phase of ARDS is characterized by the outflow of plasma and cells from the circulation to the extravascular tissues setting up alveolar edema, mediated by inflammatory cytokines(Thompson, Chambers et al. 2017), (Sweeney and McAuley 2016) .

Without therapeutic intervention, this process can cause irreversible damage to the lungs. Matrix metalloproteinases (MMPs) play an important role in innate immunity and inflammatory response, and various inflammatory lung diseases such as ARDS, asthma, and pulmonary fibrosis are characterized by an increase in the expression of one or more of the MMPs (Nagase, Visse et al. 2006). MMP3, also known as stromelysin1 is a matricellular protease that not only degrades the extracellular matrix in the basement membrane but also promotes the cell-cell barrier disruption due to its enzymatic proteolysis of junctional proteins, particularly the tight junction proteins claudins and occludins(Sternlicht and Werb 2001).

Endothelial cells (Artham, Gao et al. 2019), (Artham, Verma et al. 2020) bronchial and alveolar epithelial cells and inflammatory cells (Yamashita, Dolgonos et al. 2011) are reported to secrete MMP3 in the lungs, suggesting its potential role in the early stages of ARDS. Notably, MMP3 inhibition reversed MMP3s association with inflammation via increased interleukin-1β and monocyte chemoattractant protein-3 expression (Nagase, Visse et al. 2006). In agreement with the finding that MMP3 is associated with inflammation and lung injury, studies from our laboratory have demonstrated that FoxO-regulated expression of MMP3 in endothelial cells promotes bacterial lipopolysaccharide (LPS)-induced endothelial-barrier disruption in vitro and lung injury and edema in vivo (Artham, Gao et al. 2019). Increased endothelial MMP3 levels in our studies correlated with decreased expression of predominant endothelial tight junction protein claudin-5, whose decrease is associated with ARDS, suggesting the potential role of MMP3 in the promotion of vascular permeability (Schlingmann, Molina et al. 2015). Further, LPS-induced lung injury also correlated with increased expression of MMP3 in the bronchoalveolar lavage fluid (BALF) associated with increased neutrophil myeloperoxidase activity in the plasma, lung tissue, and the BALF (Artham, Gao et al. 2019). Another study from our group confirmed this further in

human ARDS patients were significantly elevated MMP3 activity was observed in the plasma/serum samples of ARDS patients compared to the samples collected from the healthy control subjects (Artham, Verma et al. 2020). The mechanisms by which MMP3 expression is regulated in inflammatory cells are currently unknown. Nevertheless, co-treatment with MMP3 inhibitor UK356618 effectively reversed LPS-induced endothelial-barrier disruption in vitro and lung injury and edema in vivo (Artham, Gao et al. 2019) indicating the role of MMP3 in mediating pathological inflammation and tissue injury.

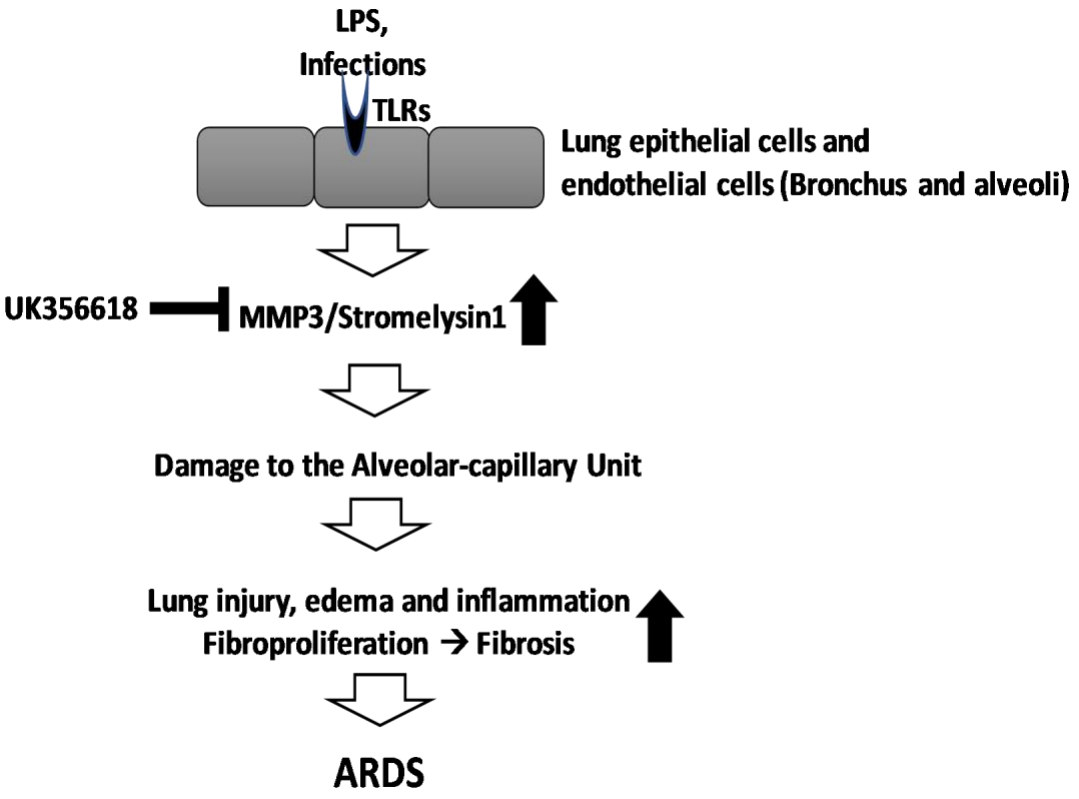

Figure 1: MMP3 inhibition during the exudative phase of ARDS reduces vascular injury and alveolar edema, and prevent the disease progression to the irreversible stages of the disease. During ARDS, there is a potential risk of increased MMP3 expression and activation in the lung epithelial and endothelial cells resulting in injury to the alveolar-capillary unit. Inhibition of MMP3 activity by treatment with UK356618 may have potential therapeutic benefits in reducing the damage to the alveolar-capillary unit thus preventing lung edema and inflammation.

Beyond the potential therapeutic benefits of targeting MMP3 in COVID-19, the measurement of MMP3 activity in COVID-19 patients may also have utility as a prognostic marker, which has been demonstrated in other disease states. Previous studies have determined that the cutoff value used for Kaplan Meier survival risk shows an association between MMP3 expression and severity of cardiovascular disease(Guizani, Zidi et al. 2019). A recent study conducted in a cohort of male patients with and without acute coronary syndrome used MMP3 expression level to predict myocardial infarction (MI) (Cavusoglu, Marmur et al. 2016). In pulmonary fibrosis, a correlation between the expression of MMP3, endostatin, inflammatory cytokines in BALF and lung functionality of IPF patients has been established(Craig, Zhang et al. 2015). Although a similar study has yet to be undertaken in ARDS or COVID-19 patients, our recent report on the elevated MMP3 activity in the serum/plasma samples of ARDS patients is an indication that MMP3 activity measurement may serve as an important prognosis marker for the disease severity of COVID-19 patients.

1.9. Mechanisms of MMP3 expression regulation.

The upstream signaling pathway that regulates the MMP3 expression in the endothelial cells and the lungs identified to be the Akt1-forkhead box-O transcription factors 1/3a (FoxO 1/3a)-MMP3 pathway in am LPS-induced experimental ALI model (Artham, Gao et al. 2019) (Tam, Fedoseyeva et al. 1996) (Wang, Zhou et al. 2014), The inhibition of AKT-1 action by LPS in pulmonary endothelial cells resulted in the increased activity and expression of FoxO1/3a and MMP3 associated with reduced expression of tight junction proteins, particularly claudin-5 (Jang, Concel et al. 2011). This resulted in the endothelial barrier breakdown, subsequently causing vascular leakage and edema (Artham, Gao et al. 2019), an effect that was reversed by the pharmacological inhibition of MMP3 or FoxO.

1.10. The effect of cisatracurium on experimental ALI

Recent studies showed the effect of cisatracurium in attenuating LPS-induced ALI in mice via inhibition of inflammation through reduced nicotinic acetylcholine receptor-α1 (nAChRα1) activity in a dose-dependent manner (Fanelli, Morita et al. 2016). nAChRα receptor is expressed in most of lung cell types, endothelial, epithelial cells, neutrophils, macrophages, and fibroblasts and has been identified as a functional cell receptor for urokinase, studies showed that it's elevated in ALI and increase pulmonary vascular permeability (Makarova, Lebedeva et al. 2011). Under normal conditions, upon binding to the nAChRα1 receptor, conformational changes allow passage of sodium and calcium ions causing calpain activation, in turn, mediating the NF-KB pathway through nuclear translocation and initiation the inflammatory response (Zdanowski, Krzyzowska et al. 2015). It was found that Cisatracurium has anti-inflammatory effects independent of its synchronizing effect to protect against ventilation-induced ALI (Fanelli, Morita et al. 2016). Cisatracurium was also investigated in a colorectal cancer model, to decrease invasion and retard migration by upregulation of P53 protein expression and activity, resulting in decreased colorectal cancer progression and aggressiveness. Cisatracurium inhibited tumor cell growth and metastasis, by upregulating of P53 and its downstream pathway proteins responsible for proliferation in the in vitro and in vivo model (Yabasin, Sanches et al. 2018). Apart from these, cisatracurium demonstrated an increase in E-cadherin expression and reduced SNAI-1 and SLUG levels thus preventing epithelial-to-mesenchymal transition (EMT) (Wang 2001, Mukhopadhyay, Eves et al. 2009, Wang, Wang et al. 2009). The upstream and downstream mechanistic pathways for cisatracurium in different cells and disease models need further investigation.

The objective, central hypothesis, and specific aims

The Matrix metalloproteinases (MMPs) are proteolytic enzymes that can degrade the extracellular matrix (ECM) components, and play an important role in the innate immunity and inflammatory response. Various inflammatory lung diseases such as ARDS, asthma, and pulmonary fibrosis are characterized by an increase in the expression of one or more of the MMPs. Recent studies in our laboratory have identified the therapeutic benefits of targeting MMP3 (stromelysin1) as well as measuring MMP3 activity in biological fluids such as bronchoalveolar lavage fluid (BALF) as a reliable biomarker for acute lung injury in a lipopolysaccharide-induced model of mouse acute lung injury and cellular studies. Currently, there is a ***gap in our knowledge*** on the protective and the anti-inflammatory pathways regulated by Cisatracurium in lung endothelial and epithelial cells, that could act the same way in ARDS patients. The ***objective*** of the current proposal is to determine if cisatracurium affects the levels of MMP3 in human microvascular endothelial cells and lung epithelial cells *in vitro*. Our ***central hypothesis*** is that cisatracurium will protect the endothelium and lung epithelium against the inflammatory insult by protecting the barrier functionality via MMP3 expression and activity *in vitro*. *The rationale* is that our study will provide novel insights into the molecular mechanisms by which cisatracurium offers barrier protection.

I will test my central hypothesis by pursuing the following two *specific aims:*

Aim1: Investigate the endothelial and epithelial protective benefits of cisatracurium.

Aim2: Determine the effect of cisatracurium on MMP3 expression and activity in endothelial cells and lung epithelial cells *in vitro*.

References:

1. Alexander, J. S. and J. W. Elrod (2002). "Extracellular matrix, junctional integrity and matrix metalloproteinase interactions in endothelial permeability regulation." J Anat **200**(6): 561-574.

2. Artham, S., F. Gao, A. Verma, A. Alwhaibi, H. Sabbineni, S. Hafez, A. Ergul and P. R. Somanath (2019). "Endothelial stromelysin1 regulation by the forkhead box-O transcription factors is crucial in the exudative phase of acute lung injury." Pharmacol Res **141**: 249-263.

3. Bellani, G., J. G. Laffey, T. Pham, E. Fan, L. Brochard, A. Esteban, L. Gattinoni, F. van Haren, A. Larsson, D. F. McAuley, M. Ranieri, G. Rubenfeld, B. T. Thompson, H. Wrigge, A. S. Slutsky, A. Pesenti, L. S. Investigators and E. T. Group (2016). "Epidemiology, Patterns of Care, and Mortality for Patients With Acute Respiratory Distress Syndrome in Intensive Care Units in 50 Countries." JAMA **315**(8): 788-800.

4. Cavusoglu, E., J. D. Marmur, J. T. Kassotis, S. Yanamadala, V. Chopra and C. Eng (2016). "Usefulness of Plasma Matrix Metalloproteinase-3 Levels to Predict Myocardial Infarction in Men With and Without Acute Coronary Syndrome." Am J Cardiol **117**(6): 881-886.

5. Choi, D. H., O. Hwang, K. H. Lee, J. Lee, M. F. Beal and Y. S. Kim (2011). "DJ-1 cleavage by matrix metalloproteinase 3 mediates oxidative stress-induced dopaminergic cell death." Antioxid Redox Signal **14**(11): 2137-2150.

6. Fanelli, V., Y. Morita, P. Cappello, M. Ghazarian, B. Sugumar, L. Delsedime, J. Batt, V. M. Ranieri, H. Zhang and A. S. Slutsky (2016). "Neuromuscular Blocking Agent Cisatracurium Attenuates Lung Injury by Inhibition of Nicotinic Acetylcholine Receptor-alpha1." Anesthesiology **124**(1): 132-140.

7. Fernandez, A. L., M. Koval, X. Fan and D. M. Guidot (2007). "Chronic alcohol ingestion alters claudin expression in the alveolar epithelium of rats." Alcohol **41**(5): 371-379.

8. Frantz, C., K. M. Stewart and V. M. Weaver (2010). "The extracellular matrix at a glance." J Cell Sci **123**(Pt 24): 4195-4200.

9. Greenberg, S. B. and J. Vender (2013). "The use of neuromuscular blocking agents in the ICU: where are we now?" Crit Care Med **41**(5): 1332-1344.

10. Guerin, C., J. Reignier, J. C. Richard, P. Beuret, A. Gacouin, T. Boulain, E. Mercier, M. Badet, A. Mercat, O. Baudin, M. Clavel, D. Chatellier, S. Jaber, S. Rosselli, J. Mancebo, M. Sirodot, G. Hilbert, C. Bengler, J. Richecoeur, M. Gainnier, F. Bayle, G. Bourdin, V. Leray, R. Girard, L. Baboi, L. Ayzac and P. S. Group (2013). "Prone positioning in severe acute respiratory distress syndrome." N Engl J Med **368**(23): 2159-2168.

11. Guizani, I., W. Zidi, Y. Zayani, S. Boudiche, S. Hadj-Taieb, H. Sanhaji, A. Zaroui, R. Mechmeche, M. S. Mourali, M. Feki and M. Allal-Elasmi (2019). "Matrix metalloproteinase-3 predicts clinical cardiovascular outcomes in patients with coronary artery disease: a 5 years cohort study." Mol Biol Rep **46**(5): 4699-4707.

12. Hall, J. B. (2013). "Point: Should paralytic agents be routinely used in severe ARDS? Yes." Chest **144**(5): 1440-1442.

13. Huang, D. T., D. C. Angus, M. Moss, B. T. Thompson, N. D. Ferguson, A. Ginde, M. N. Gong, S. Gundel, D. L. Hayden, R. D. Hite, P. C. Hou, C. L. Hough, T. J. Iwashyna, K. D. Liu, D. S. Talmor, D. M. Yealy, C. Reevaluation of Systemic Early Neuromuscular Blockade Protocol, L. the National Institutes of Health National Heart, P. Blood Institute and I. Early Treatment of Acute Lung Injury Network (2017). "Design and Rationale of the Reevaluation of Systemic Early Neuromuscular Blockade Trial for Acute Respiratory Distress Syndrome." Ann Am Thorac Soc **14**(1): 124-133.

14. Jablonska-Trypuc, A., M. Matejczyk and S. Rosochacki (2016). "Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs." J Enzyme Inhib Med Chem **31**(sup1): 177-183.

15. Koval, M. (2013). "Claudin heterogeneity and control of lung tight junctions." Annu Rev Physiol **75**: 551-567.

16. Lech, A. M., G. Wiera and J. W. Mozrzymas (2019). "Matrix metalloproteinase-3 in brain physiology and neurodegeneration." Adv Clin Exp Med **28**(12): 1717-1722.

17. Makarova, A. M., T. V. Lebedeva, T. Nassar, A. A. Higazi, J. Xue, M. E. Carinato, K. Bdeir, D. B. Cines and V. Stepanova (2011). "Urokinase-type plasminogen activator (uPA) induces pulmonary microvascular endothelial permeability through low density lipoprotein

receptor-related protein (LRP)-dependent activation of endothelial nitric-oxide synthase." J Biol Chem **286**(26): 23044-23053.

18. Matthay, M. A., R. L. Zemans, G. A. Zimmerman, Y. M. Arabi, J. R. Beitler, A. Mercat, M. Herridge, A. G. Randolph and C. S. Calfee (2019). "Acute respiratory distress syndrome." Nat Rev Dis Primers **5**(1): 18.

19. Mukhopadhyay, U. K., R. Eves, L. Jia, P. Mooney and A. S. Mak (2009). "p53 suppresses Src-induced podosome and rosette formation and cellular invasiveness through the upregulation of caldesmon." Mol Cell Biol **29**(11): 3088-3098.

20. Nagase, H., R. Visse and G. Murphy (2006). "Structure and function of matrix metalloproteinases and TIMPs." Cardiovasc Res **69**(3): 562-573.

21. National Heart, L., P. C. T. N. Blood Institute, M. Moss, D. T. Huang, R. G. Brower, N. D. Ferguson, A. A. Ginde, M. N. Gong, C. K. Grissom, S. Gundel, D. Hayden, R. D. Hite, P. C. Hou, C. L. Hough, T. J. Iwashyna, A. Khan, K. D. Liu, D. Talmor, B. T. Thompson, C. A. Ulysse, D. M. Yealy and D. C. Angus (2019). "Early Neuromuscular Blockade in the Acute Respiratory Distress Syndrome." N Engl J Med **380**(21): 1997-2008.

22. Papazian, L., C. Aubron, L. Brochard, J. D. Chiche, A. Combes, D. Dreyfuss, J. M. Forel, C. Guerin, S. Jaber, A. Mekontso-Dessap, A. Mercat, J. C. Richard, D. Roux, A. Vieillard-Baron and H. Faure (2019). "Formal guidelines: management of acute respiratory distress syndrome." Ann Intensive Care **9**(1): 69.

23. Papazian, L., J. M. Forel, A. Gacouin, C. Penot-Ragon, G. Perrin, A. Loundou, S. Jaber, J. M. Arnal, D. Perez, J. M. Seghboyan, J. M. Constantin, P. Courant, J. Y. Lefrant, C. Guerin, G. Prat, S. Morange, A. Roch and A. S. Investigators (2010). "Neuromuscular blockers in early acute respiratory distress syndrome." N Engl J Med **363**(12): 1107-1116.

24. Sun, S., A. C. Bay-Jensen, M. A. Karsdal, A. S. Siebuhr, Q. Zheng, W. P. Maksymowych, T. G. Christiansen and K. Henriksen (2014). "The active form of MMP-3 is a marker of synovial inflammation and cartilage turnover in inflammatory joint diseases." BMC Musculoskelet Disord **15**: 93.

25. Sweeney, R. M. and D. F. McAuley (2016). "Acute respiratory distress syndrome." Lancet **388**(10058): 2416-2430.

26. Thompson, B. T., R. C. Chambers and K. D. Liu (2017). "Acute Respiratory Distress Syndrome." N Engl J Med **377**(6): 562-572.

27. Van Hove, I., K. Lemmens, S. Van de Velde, M. Verslegers and L. Moons (2012). "Matrix metalloproteinase-3 in the central nervous system: a look on the bright side." J Neurochem **123**(2): 203-216.

28. Wallez, Y. and P. Huber (2008). "Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis." Biochim Biophys Acta **1778**(3): 794-809.

29. Wang, C. L. (2001). "Caldesmon and smooth-muscle regulation." Cell Biochem Biophys **35**(3): 275-288.

30. Wang, S. P., W. L. Wang, Y. L. Chang, C. T. Wu, Y. C. Chao, S. H. Kao, A. Yuan, C. W. Lin, S. C. Yang, W. K. Chan, K. C. Li, T. M. Hong and P. C. Yang (2009). "p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug." Nat Cell Biol **11**(6): 694-704.

31. Ward, C., B. Schlingmann, A. A. Stecenko, D. M. Guidot and M. Koval (2015). "NF-kappaB inhibitors impair lung epithelial tight junctions in the absence of inflammation." Tissue Barriers **3**(1-2): e982424.

32. Warr, J., Z. Thiboutot, L. Rose, S. Mehta and L. D. Burry (2011). "Current therapeutic uses, pharmacology, and clinical considerations of neuromuscular blocking agents for critically ill adults." Ann Pharmacother **45**(9): 1116-1126.

33. Yabasin, I. B., J. G. P. Sanches, M. M. Ibrahim, J. Huidan, W. Williams, Z. L. Lu and Q. Wen (2018). "Cisatracurium Retards Cell Migration and Invasion Upon Upregulation of p53 and Inhibits the Aggressiveness of Colorectal Cancer." Front Physiol **9**: 941.

34. Renew, J. R., R. Ratzlaff, V. Hernandez-Torres, S. J. Brull and R. C. Prielipp (2020). "Neuromuscular blockade management in the critically Ill patient." J Intensive Care **8**: 37.

35. Shah, F. A., T. D. Girard and S. Yende (2017). "Limiting sedation for patients with acute respiratory distress syndrome - time to wake up." Curr Opin Crit Care **23**(1): 45-51.

36. Pearson, S. D. and B. K. Patel (2020). "Evolving targets for sedation during mechanical ventilation." Curr Opin Crit Care **26**(1): 47-52.

37. Bourenne, J., S. Hraiech, A. Roch, M. Gainnier, L. Papazian and J. M. Forel (2017). "Sedation and neuromuscular blocking agents in acute respiratory distress syndrome." Ann Transl Med **5**(14): 291.

38. Murray, M. J., H. DeBlock, B. Erstad, A. Gray, J. Jacobi, C. Jordan, W. McGee, C. McManus, M. Meade, S. Nix, A. Patterson, M. K. Sands, R. Pino, A. Tescher, R. Arbour,

B. Rochwerg, C. F. Murray and S. Mehta (2016). "Clinical Practice Guidelines for Sustained Neuromuscular Blockade in the Adult Critically Ill Patient." Crit Care Med **44**(11): 2079-2103.

39. Blondonnet, R., J. M. Constantin, V. Sapin and M. Jabaudon (2016). "A Pathophysiologic Approach to Biomarkers in Acute Respiratory Distress Syndrome." Dis Markers **2016**: 3501373.

40. Walkey, A. J., R. Summer, V. Ho and P. Alkana (2012). "Acute respiratory distress syndrome: epidemiology and management approaches." Clin Epidemiol **4**: 159-169.

41. Potey, P. M., A. G. Rossi, C. D. Lucas and D. A. Dorward (2019). "Neutrophils in the initiation and resolution of acute pulmonary inflammation: understanding biological function and therapeutic potential." J Pathol **247**(5): 672-685.

42. Han, S. and R. K. Mallampalli (2015). "The acute respiratory distress syndrome: from mechanism to translation." J Immunol **194**(3): 855-860.

43. Gonzales, J. N., R. Lucas and A. D. Verin (2015). "The Acute Respiratory Distress Syndrome: Mechanisms and Perspective Therapeutic Approaches." Austin J Vasc Med **2**(1).

44. Han, S. and R. K. Mallampalli (2015). "The acute respiratory distress syndrome: from mechanism to translation." J Immunol **194**(3): 855-860.

45. Zdanowski, R., M. Krzyzowska, D. Ujazdowska, A. Lewicka and S. Lewicki (2015). "Role of alpha7 nicotinic receptor in the immune system and intracellular signaling pathways." Cent Eur J Immunol **40**(3): 373-379.

46. Tam, R. C., E. V. Fedoseyeva, M. Moskalenko, M. R. Garovoy and G. Benichou (1996). "T cell tolerance is influenced by concomitant T cell recognition of cross-reactive self-peptides." J Immunol **156**(10): 3765-3771.

47. Wang, Y., Y. Zhou and D. T. Graves (2014). "FOXO Transcription Factors: Their Clinical Significance and Regulation." BioMed Research International **2014**: 925350.

48. Jang, A. S., V. J. Concel, K. Bein, K. A. Brant, S. Liu, H. Pope-Varsalona, R. A. Dopico, Jr., Y. P. Di, D. L. Knoell, A. Barchowsky and G. D. Leikauf (2011). "Endothelial dysfunction and claudin 5 regulation during acrolein-induced lung injury." Am J Respir Cell Mol Biol **44**(4): 483-490.

49. Guizani, I., W. Zidi, Y. Zayani, S. Boudiche, S. Hadj-Taieb, H. Sanhaji, A. Zaroui, R. Mechmeche, M. S. Mourali, M. Feki and M. Allal-Elasmi (2019). "Matrix metalloproteinase-3 predicts clinical cardiovascular outcomes in patients with coronary artery disease: a 5 years cohort study." Mol Biol Rep **46**(5): 4699-4707.

50. Cavusoglu, E., J. D. Marmur, J. T. Kassotis, S. Yanamadala, V. Chopra and C. Eng (2016). "Usefulness of Plasma Matrix Metalloproteinase-3 Levels to Predict Myocardial Infarction in Men With and Without Acute Coronary Syndrome." Am J Cardiol **117**(6): 881-886.

51. Craig, V. J., L. Zhang, J. S. Hagood and C. A. Owen (2015). "Matrix metalloproteinases as therapeutic targets for idiopathic pulmonary fibrosis." Am J Respir Cell Mol Biol **53**(5): 585-600.

52. Greenlee, K. J., Z. Werb and F. Kheradmand (2007). "Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted." Physiol Rev **87**(1): 69-98.

53. Loffek, S., O. Schilling and C. W. Franzke (2011). "Series "matrix metalloproteinases in lung health and disease": Biological role of matrix metalloproteinases: a critical balance." Eur Respir J **38**(1): 191-208.

54. Artham, S., A. Verma, A. S. Newsome and P. R. Somanath (2020). "Patients with acute respiratory distress syndrome exhibit increased stromelysin1 activity in the blood samples." Cytokine **131**: 155086.

55. Schlingmann, B., S. A. Molina and M. Koval (2015). "Claudins: Gatekeepers of lung epithelial function." Semin Cell Dev Biol **42**: 47-57.

56. Sternlicht, M. D. and Z. Werb (2001). "How matrix metalloproteinases regulate cell behavior." Annu Rev Cell Dev Biol **17**: 463-516.

57. Yamashita, C. M., L. Dolgonos, R. L. Zemans, S. K. Young, J. Robertson, N. Briones, T. Suzuki, M. N. Campbell, J. Gauldie, D. C. Radisky, D. W. Riches, G. Yu, N. Kaminski, C. A. McCulloch and G. P. Downey (2011). "Matrix metalloproteinase 3 is a mediator of pulmonary fibrosis." Am J Pathol **179**(4): 1733-1745.

58. Bonnans, C., J. Chou and Z. Werb (2014). "Remodelling the extracellular matrix in development and disease." Nat Rev Mol Cell Biol **15**(12): 786-801.

59. Zhang, Y. and W. X. Yang (2016). "Tight junction between endothelial cells: the interaction between nanoparticles and blood vessels." Beilstein J Nanotechnol **7**: 675-684.
60. Rundhaug, J. E. (2005). "Matrix metalloproteinases and angiogenesis." J Cell Mol Med **9**(2): 267-285.

61. Herrero, R., G. Sanchez and J. A. Lorente (2018). "New insights into the mechanisms of pulmonary edema in acute lung injury." Ann Transl Med **6**(2): 32.

62. Matthay, M. A., L. B. Ware and G. A. Zimmerman (2012). "The acute respiratory distress syndrome." J Clin Invest **122**(8): 2731-2740.

63. Whitsett, J. A., S. E. Wert and T. E. Weaver (2010). "Alveolar surfactant homeostasis and the pathogenesis of pulmonary disease." Annu Rev Med **61**: 105-119.

64. Herzog, E. L., A. R. Brody, T. V. Colby, R. Mason and M. C. Williams (2008). "Knowns and unknowns of the alveolus." Proc Am Thorac Soc **5**(7): 778-782.

65. Koval, M. (2013). "Claudin heterogeneity and control of lung tight junctions." Annu Rev Physiol **75**: 551-567.

66. Carra, S., E. Foglia, S. Cermenati, E. Bresciani, C. Giampietro, C. Lora Lamia, E. Dejana, M. Beltrame and F. Cotelli (2012). "Ve-ptp modulates vascular integrity by promoting adherens junction maturation." PLoS One **7**(12): e51245.

Chapter 2

Cisatracurium attenuates LPS-induced increase in MMP3 expression in microvascular endothelial cells and lung epithelial cells[1]

[1]***Kadry, Rana W. et al.,*** **Manuscript to be submitted to the *Journal of Cellular Physiology*.**

Cisatracurium attenuates LPS-induced increase in MMP3 expression in microvascular endothelial cells and lung epithelial cells

ABSTRACT

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are associated with inflammation, hypoxemia, and respiratory failure leading to high morbidity and mortality. Previous studies have implicated matrix metalloproteinase protein-3 (MMP3) as increasing ALI/ARDS and that its pharmacological inhibition reverses bacterial endotoxin-induced ALI in mice. Cisatracurium, a neuromuscular blocking agent (NMBA), is used to manage mechanically ventilated ARDS patients to improve oxygenation and reduce ventilator-induced lung injury; however, a gap exists in the knowledge of how cisatracurium improves patient outcomes. The objective of the current study is to investigate the effect of cisatracurium to modulate MMP3 expression/activity in microvascular endothelial cells and lung epithelial cells and further its ability to prevent lipopolysaccharide (LPS)-induced cell-monolayer injury. In our results, cisatracurium decreased MMP3 expression/activity and increased expression of cell junction proteins suggesting endothelial- and epithelial-barrier protective activity.

KEYWORDS: Cisatracurium; MMP3; Stromelysin1; lipopolysaccharide; cell junction

Introduction

Acute respiratory distress syndrome (ARDS) results in a patient's inability to oxygenate effectively due to lung injury from diffuse alveolar damage and subsequent edema (Matthay, Zemans, et al. 2019). Lack of effective diagnostic methods frequently results in delayed diagnosis, which is associated with high morbidity and mortality (Bellani, Laffey, et al. 2016). Since the 1980s, several clinical trials have been conducted to develop strategies to manage ARDS patients with minimal success (Cepkova and Matthay 2006). The updated guidelines for ARDS management include only supportive care treatments (e.g., mechanical ventilation) (Papazian, Aubron, et al. 2019). Neuromuscular blocking agents (NMBAs) are used in supportive care to decrease patient-ventilator dyssynchrony in mechanically ventilated ARDS patients (Hall 2013). Cisatracurium has the most evidence for its use in ARDS (Murray, DeBlock, et al. 2016). The efficacy of cisatracurium was studied in two large clinical trials (ACURASYS and ROSE) (National Heart, Blood Institute et al. 2019), (Papazian, Forel, et al. 2010).

The clinical progression of ARDS has three phases: Exudative phase, Proliferative phase, and Fibrotic phase (Thompson, Chambers, et al. 2017). Among these, the exudative stage of ARDS is characterized by diffuse alveolar damage accompanied by lung inflammation and edema (Walkey, Summer, et al. 2012). At the cellular level, the exudative stage of ARDS is due to the extensive damage to the alveolar epithelium and the capillary endothelium, which in the normal stages are tightly connected by the cell-cell junctions (Herrero, Sanchez, et al. 2018). Damage to the alveolar-capillary unit results in neutrophil infiltration and edema (Han and Mallampalli 2015).

Hence, preventing injury to the alveolar-capillary unit may be a strategy to halt ARDS disease progression. The junctional protein complexes between the cells in epithelial or endothelial monolayers play a crucial role in the selective air-blood barrier integrity of the lungs (Matthay, Zemans, et al. 2019). In ARDS damaged lungs, the barrier integrity is compromised leading to fluid leakage into the alveolar air spaces causing lung edema (Gonzales, Lucas, et al. 2015). There are two types of cell junctions that maintain the barrier integrity namely adherens junctions (AJ) and tight junctions (TJ) (Overgaard, Mitchell, et al. 2012), (Koval 2013). Research in our laboratory has demonstrated the integral role of the Akt pathway in endothelial-barrier protection (Chen, Somanath, et al. 2005), (Gao, Sabbineni, et al. 2017) by removing the transcriptional suppression by FoxO and β-catenin (Gao, Sabbineni et al. 2017) (Alwhaibi, Verma et al. 2019). Inhibition of Akt pathway resulted in the reduced expression of TJ proteins, mainly claudin-5 (Gao, Artham, et al. 2016) in the endothelial cells and the lung blood vessels, leading lung injury and edema (Artham, Gao et al. 2019) and ultimately endothelial-to-mesenchymal transition (Sabbineni, Verma et al. 2019).

Beyond the transcriptional suppression of TJ proteins, activated FoxO was also observed to increase the expression of matrix metalloproteinases-3 (MMP3/Stromolysin-1) (Artham, Gao, et al. 2019), a protease that is previously shown to damage the junctional protein complexes (Nagase, Visse et al. 2006). Increased expression and activity of MMP3 in the LPS-injured lungs and bronchoalveolar lavage fluid (BALF) in mice (Artham, Gao, et al. 2019) and ARDS patient plasma samples (Artham, Verma et al. 2020) has also been observed. In the current study, we investigated the direct effect of cisatracurium to suppress LPS-induced MMP3 expression in human microvascular endothelial cells and lung epithelial cells in protecting the endothelial and epithelial barrier integrity. In our analysis, the direct effects of cisatracurium on endothelial and

epithelial barrier protein expression were modest. Nevertheless, our study demonstrated the ability of cisatracurium in reducing the expression of LPS-induced MMP3 by the endothelial and epithelial cells.

Materials and Methods:

1. Cell culture

Human dermal (Telomerase-immortalized) microvascular ECs (HMECs) (CRL-4025; ATCC, Manassas, VA) were maintained in EC Basal Medium-2 with a Growth Medium-2 Bullet Kit (Lonza; Walkersville, MD) and alveoli epithelial cells (A549) was cultured in DMEM medium, human bronchial epithelial cells (H441) was cultured in RPMI media from hyclone. All cultures were maintained in a humidified 5% CO_2 incubator at 37 °C and routinely passaged when 80–90% confluent. Cisatracurium (cat. No S2113, Selleckchem) was reconstituted according to the manufacturer's protocol. HMECs, A549, and H441celllines were treated with 100 ng/ml LPS and different doses of cisatracurium 0.32, 0.64, and 1.28μM and PBS (vehicle), respectively, in 5% serum-containing medium for 24 hours. The growth factors were replenished every 24 hours. The optimal doses of cisatracurium were determined based on a similar study (Fanelli, Morita, et al. 2016).

2. Stromelysin1 activity assay

The enzymatic activity of stromelysin1 was determined using a fluorescence resonance energy transfer (FRET) peptide and immunocapture assay as previously described (Artham, Gao, et al. 2019). Briefly, 50 μg total protein of cell lysates were incubated at 4°C for 2 hours with rabbit polyclonal anti-MMP3 antibody (cat no. sc-21732-Mouse monoclonal IgG; Santa Cruz Biotechnology, Dallas, TX). A/G agarose beads were then added and allowed to incubate

overnight at 4°C. The beads were then washed, and samples were transferred to a black 96well plate and 100 μL of 2 μmol/L 5-FAM/QXL 520 FRET peptide (cat no. 60580-01; AnaSpec, San Jose, CA) in assay buffer was added per well. Plates were incubated for 15 hours at 37°C; then relative fluorescence units were read and monitored at excitation/emission wavelengths of 485 of 528 nm in a Synergy HT multimode microplate fluorescence reader (BioTek, Winooski, VT) running Gen5 data analysis software.

3. MTT assay

Cell proliferation and viability were determined as previously published from our laboratory using the MTT assay (Al-Azayzih, Missaoui, Cummings, & Somanath, 2016). Cells were seeded in 48-well cell culture plates at 5×10^4 cells/ml and incubated at 37 °C in a 5% CO_2 incubator for 24 hours. Cells were treated with 100 ng/ml LPS and different doses of cisatracurium 0.32, 0.64, and 1.28μM and PBS (vehicle) and were incubated for 24 hours. MTT was added at this time point, at a final concentration of 0.25 mg/ml and plates were incubated at 37 °C. Non-reduced MTT and media were aspirated after 2 hours and replaced with DMSO to dissolve the MTT formazan crystals. Plates were shaken for 15 minutes and absorbance was read at 590 nm using a Biotek plate reader (Biotek, Winooski, VT).

4. Trypan blue viability assessment

In the trypan blue method, H441 cells were grown to confluence in RPMI medium with 10% FBS. The cells were treated with Cisatracurium 0.32, 0.64, and 1.28μM and PBS (vehicle). After 24 hours, cells were collected and re-suspended in PBS with 0.4% Trypan blue solution. Total cells and Trypan blue stained (i.e., non-viable) cells were counted and the percentage of non-viable cells was calculated.

5. Western blot analysis

Cell lysates were prepared using complete lysis buffer (EMD Millipore, San Diego, CA) with protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein quantification was performed using DC protein assay from Bio-Rad (Hercules, and CA). Western blot analysis was performed as described previously (Sabbineni, Verma, et al. 2018), (Goc, Al-Azayzih, et al. 2013). Antibodies used include stromelysin1 (cat. No. 14351-S) dilution 1:1000 in milk, E-cadherin (cat No. 4065-S) dilution 1:1000 in BSA and VE-cadherins (cat No. 2158) dilution 1:1000 in BSA, P-P38 MAPK (cat No. 9112-S) dilution 1:1000 in BSA, T-P38MAPK (cat No.9212-S) dilution 1:1000 in BSA, P-SRC Tyr-416 (cat No. 6943-S) dilution 1:1000 in BSA and T-SRC (cat No. 2109-S) dilution 1:1000 in BSA all from Cell Signaling Technology (Danvers, MA). β-actin (dilution in milk, primary antibodies 1:10000 and secondary antibodies 1:20000) from Sigma (St. Louis, MO) and Claudin-5 antibodies (cat No. ab15106) 1:1000 and secondary antibodies 1:5000 dilution in milk from Abcam (Cambridge, MA). Band densitometry was done using NIH Image J software.

6. Statistical Analysis

All the data are presented as mean ± SD and were calculated from multiple independent experiments performed in triplicates. The ‘n’ value for each figure implies the multiple independent experiments we performed. All the data were analyzed by parametric testing using the Student's unpaired t-test or one-way ANOVA, followed by the posthoc test using the GraphPad Prism 6.01 software. Data with $P<0.05$ were considered significant.

Results

1. Treatment with cisatracurium reduced LPS-induced increase in MMP3 expression and activity

To test the effect of cisatracurium in inhibiting the LPS-induced expression of MMP3, we treated human microvascular endothelial cells (HMECs) and human bronchial epithelial (H441) cells with 100 ng/ml of LPS and three different doses of cisatracurium for 24 hours. Following this, Western blotting of the cell lysates and band densitometry analysis were performed to determine the expression of MMP3 normalized to the β-actin expression (housekeeping gene as a loading control). Our results indicated a significantly higher MMP3 expression in HMECs and H441 cells with LPS treatment for 24 hours, which was blunted by co-treatment with cisatracurium (Figure 1A-B). We also performed MMP3 activity assay in LPS and/or cisatracurium-treated alveolar epithelial (A549) cells using a FRET technique. Similar to the HMECs and H441 cells, our results indicated an increase in MMP3 activity upon 24 hours treatment with LPS and its inhibition by co-treatment with cisatracurium (Figure 2). However, MMP3 expression in A549 cells was below detectable levels on a Western blot. Our results indicated that cisatracurium blunts LPS-induced MMP3 expression/activity in endothelial and lung epithelial cells.

2. Treatment with cisatracurium reduced LPS-induced increase in Src phosphorylation

Src has been demonstrated to break up adherens junction cell interactions through the cadherins (Dejana, Orsenigo, et al. 2008). Next, we determined how LPS and cisatracurium treatment effect Src activating phosphorylation at Tyrosine-416 residue. Our analysis indicated that both in

HMECs and H441 cells, treatment with LPS for 24 hours resulted in increased levels of active Src, which was significantly inhibited by co-treatment with cisatracurium (Figure 3A-B), thus indicating that cisatracurium protects the cell-barriers by inhibiting Src activity.

3. Treatment with cisatracurium rescued LPS-induced loss of Claudin-5 and VE-Cadherin

To determine if cisatracurium could preserve AJ and TJ complexes in HMECs and H441 cells, we treated these cells with LPS and cisatracurium alone or in combination for 24 hours and subjected for the Western analysis of AJ cadherins (E-cadherin in H441 and VE-cadherin in HMECs) and TJ claudin-5. Our analysis indicated that 24 hours of treatment with LPS significantly reduced claudin-5 in HMECs, which was reversed upon co-treatment with cisatracurium (Figure 4A). Interestingly, in H441 cells, treatment with LPS increased expression of claudin-5 with a further increase upon co-treatment with cisatracurium (Figure 4B). Akin to claudin-5, treatment with LPS significantly reduced VE-cadherin expression in HMECs, which was reversed upon co-treatment with cisatracurium (Figure 5A). Surprisingly, no changes in the expression levels of E-cadherin was observed with either LPS or cisatracurium treatment in H441 cells (Figure 5B). We then determined changes in E-cadherin expression also in human alveolar A549 cells, and once again observed that LPS and cisatracurium do not change E-cadherin expression levels (Figure 6). Together, these results indicated that cisatracurium modulates AJ and TJ protein expression in endothelial and epithelial cells.

4. Treatment with cisatracurium has no significant effect on p38 MAP kinase activity modulation

Since P38 MAPK is a stress and inflammation associated kinase, we next determined if cisatracurium protects the endothelial and epithelial cells from cell stress associated with pro-inflammatory stimuli such as the bacterial LPS. Our results indicated that although LPS

stimulation of HMECs and H441 cells modestly increased the phosphorylated levels of P38 MAPK, the effect was not reversed upon co-treatment with cisatracurium (Figure 7A-B). The effect of LPS on P38 MAPK phosphorylation, however, was not observed in A549 cells (Figure 8). Overall, our data suggested that cisatracurium does not affect P38 MAPK activity in endothelial and epithelial cells.

5. Treatment with cisatracurium does not affect cell viability

To determine if cisatracurium has any effect on the viability of cells in general, we treated H441 epithelial cells with LPS and/or cisatracurium for 24 hours and subjected the cells for MTT and Trypan blue viability assays. Our analysis indicated that, although the H441 cell viability was modestly reduced by LPS treatment, cisatracurium co-treatment had no significant effect on cell viability (Figure 9).

Discussion

In patients with ALI and ARDS, the primary causes of mortality among critically ill patients are managed by both pharmacological or non-pharmacological supportive care (Thompson, Chambers, et al. 2017). Cisatracurium has been studied as a means to improve oxygenation in refractory respiratory failure in randomized-clinical trials; however, the mechanism of benefit is unknown with reduction of VILI through reducing patient-ventilator dyssynchrony vs. direct anti-inflammatory effects. In the current study, we investigated the effect of cisatracurium to modulate the pro-inflammatory and cell-barrier modulating pathways in HMECs

and lung epithelial cells and determined its efficacy to inhibit the injury-response elicited by bacterial LPS treatment.

Recent studies from our laboratory identified MMP3 as an important matricellular proteinase under the mechanistic regulation of the PI3-Kinase-Akt1-FoxO signaling pathway for its expression in the disruption of endothelial tight-junctions (Artham, Gao, et al. 2019). Resulted in increased endothelial permeability *in vitro* and vascular permeability in an experimental model of LPS-induced ALI in mice (Artham, Gao, et al. 2019). Our subsequent studies from data repositories, mouse BALF and human ARDS patient plasma samples revealed increased MMP3 expression in the liquid biopsies correlating to the severity of the disease compared to the samples from the healthy individuals (Artham, Gao et al. 2019), (Artham, Verma et al. 2020). In the current study, the ability of cisatracurium to reverse the LPS-induced increase in MMP3 expression suggested the potential utility of cisatracurium in preventing vascular injury. Activation of Src kinase is a known mechanism for the disruption of AJ protein interactions particularly by internalizing VE-cadherins (Dejana, Orsenigo, et al. 2008). Besides, the Src pathway activates myosin light chain kinase (MLCK) promoting actin-myosin interaction and gap formation (Gao F, 2017), (Okutani, 2006), (Lee, 2007). The ability cisatracurium to inhibit LPS-stimulated Src activity, in turn preventing the loss of AJ protein VE-Cadherin and TJ protein Claudin-5 in HMECs indicated its potential benefits in preventing pathological vascular permeability and inflammation. A similar effect in the H441 and A549 lung epithelial cells indicated the ability of cisatracurium to prevent a bronchial or alveolar epithelial injury. However, the fact that cisatracurium had no direct effect on the activity of pro-inflammatory P38 MAPK and no changes in the MTT assay suggests that cisatracurium does not utilize P38 MAPK to suppress inflammation and has no direct effect on the HMEC and lung epithelial cell proliferation or viability.

A limitation of the present study is that *in vitro* analysis regarding potential anti-inflammatory effects were not determined. Although cisatracurium did not have any effect in modulating the activity of P38 MAPK (a kinase that promotes inflammation), this finding does not rule out that cisatracurium cannot modulate inflammatory pathways *in vitro* or *in vivo*. Other potential pro-inflammatory pathways to explore in the future include JAK/STAT-3 and NF-KB signaling (Lee, 2007). NF-KB signaling also has a direct effect on vascular cell adhesion molecule 1 (VCAM1), intercellular adhesion molecule 1 (ICAM1), which are crucial in preserving vascular integrity in addition to its known role in inflammation (Taniguchi, K.,2018). Both pathways are essential for neutrophil recruitment and macrophage-associated damage in the exudative phase of ALI/ARDS (Okutani, 2006). Apart from these, the indirect effect of cisatracurium on endothelial and epithelial cells mediated through the suppression of inflammatory cells also cannot be ruled out.

In summary, although cisatracurium has indicated beneficial effects in suppressing inflammatory priming in endothelial cells and lung epithelial cells in preventing cell-barrier injury, the study has limitations being conducted in individual cell lines *in vitro* that a clear understanding of the collective effect and molecular mechanisms of cisatracurium in a disease model is lacking. Further studies in this area will provide us a better understanding of the underlying mechanisms and potential benefits of cisatracurium in the treatment of ARDS patients.

References:

1. Okutani, D., M. Lodyga, B. Han, and M. Liu (2006). "Src protein tyrosine kinase family and acute inflammatory responses." Am J Physiol Lung Cell Mol Physiol **291**(2): L129-141.

2. Bellani, G., J. G. Laffey, T. Pham, E. Fan, L. Brochard, A. Esteban, L. Gattinoni, F. van Haren, A. Larsson, D. F. McAuley, M. Ranieri, G. Rubenfeld, B. T. Thompson, H. Wrigge, A. S. Slutsky, A. Pesenti, L. S. Investigators and E. T. Group (2016). "Epidemiology, Patterns of Care, and Mortality for Patients With Acute Respiratory Distress Syndrome in Intensive Care Units in 50 Countries." JAMA **315**(8): 788-800.

3. National Heart, L., P. C. T. N. Blood Institute, M. Moss, D. T. Huang, R. G. Brower, N. D. Ferguson, A. A. Ginde, M. N. Gong, C. K. Grissom, S. Gundel, D. Hayden, R. D. Hite, P. C. Hou, C. L. Hough, T. J. Iwashyna, A. Khan, K. D. Liu, D. Talmor, B. T. Thompson, C. A. Ulysse, D. M. Yealy and D. C. Angus (2019). "Early Neuromuscular Blockade in the Acute Respiratory Distress Syndrome." N Engl J Med **380**(21): 1997-2008.

4. Papazian, L., J. M. Forel, A. Gacouin, C. Penot-Ragon, G. Perrin, A. Loundou, S. Jaber, J. M. Arnal, D. Perez, J. M. Seghboyan, J. M. Constantin, P. Courant, J. Y. Lefrant, C. Guerin, G. Prat, S. Morange, A. Roch and A. S. Investigators (2010). "Neuromuscular blockers in early acute respiratory distress syndrome." N Engl J Med **363**(12): 1107-1116.

5. Schlingmann, B., S. A. Molina and M. Koval (2015). "Claudins: Gatekeepers of lung epithelial function." Semin Cell Dev Biol **42**: 47-57.

6. Artham, S., F. Gao, A. Verma, A. Alwhaibi, H. Sabbineni, S. Hafez, A. Ergul and P. R. Somanath (2019). "Endothelial stromelysin1 regulation by the forkhead box-O transcription factors is crucial in the exudative phase of acute lung injury." Pharmacol Res **141**: 249-263.

7. Matthay, M. A., R. L. Zemans, G. A. Zimmerman, Y. M. Arabi, J. R. Beitler, A. Mercat, M. Herridge, A. G. Randolph and C. S. Calfee (2019). "Acute respiratory distress syndrome." Nat Rev Dis Primers **5**(1): 18.

8. Artham, S., A. Verma, A. S. Newsome and P. R. Somanath (2020). "Patients with acute respiratory distress syndrome exhibit increased stromelysin1 activity in the blood samples." Cytokine **131**: 155086.

9. Cepkova, M. and M. A. Matthay (2006). "Pharmacotherapy of acute lung injury and the acute respiratory distress syndrome." J Intensive Care Med **21**(3): 119-143.

10. Murray, M. J., H. DeBlock, B. Erstad, A. Gray, J. Jacobi, C. Jordan, W. McGee, C. McManus, M. Meade, S. Nix, A. Patterson, M. K. Sands, R. Pino, A. Tescher, R. Arbour, B. Rochwerg, C. F. Murray and S. Mehta (2016). "Clinical Practice Guidelines for Sustained Neuromuscular Blockade in the Adult Critically Ill Patient." Crit Care Med **44**(11): 2079-2103.

11. Hall, J. B. (2013). "Point: Should paralytic agents be routinely used in severe ARDS? Yes." Chest **144**(5): 1440-1442.

12. Thompson, B. T., R. C. Chambers and K. D. Liu (2017). "Acute Respiratory Distress Syndrome." N Engl J Med **377**(6): 562-572.

13. Herrero, R., G. Sanchez and J. A. Lorente (2018). "New insights into the mechanisms of pulmonary edema in acute lung injury." Ann Transl Med **6**(2): 32.

14. Walkey, A. J., R. Summer, V. Ho and P. Alkana (2012). "Acute respiratory distress syndrome: epidemiology and management approaches." Clin Epidemiol **4**: 159-169.

15. Han, S. and R. K. Mallampalli (2015). "The acute respiratory distress syndrome: from mechanism to translation." J Immunol **194**(3): 855-860.

16. Gonzales, J. N., R. Lucas and A. D. Verin (2015). "The Acute Respiratory Distress Syndrome: Mechanisms and Perspective Therapeutic Approaches." Austin J Vasc Med **2**(1).

17. Overgaard, C. E., L. A. Mitchell and M. Koval (2012). "Roles for claudins in alveolar epithelial barrier function." Ann N Y Acad Sci **1257**: 167-174.

18. Koval, M. (2013). "Claudin heterogeneity and control of lung tight junctions." Annu Rev Physiol **75**: 551-567.

19. Chen, J., P. R. Somanath, O. Razorenova, W. S. Chen, N. Hay, P. Bornstein and T. V. Byzova (2005). "Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo." Nat Med **11**(11): 1188-1196.

20. Gao, F., H. Sabbineni, S. Artham and P. R. Somanath (2017). "Modulation of long-term endothelial-barrier integrity is conditional to the cross-talk between Akt and Src signaling." J Cell Physiol **232**(10): 2599-2609.

21. Alwhaibi, A., A. Verma, M. S. Adil and P. R. Somanath (2019). "The unconventional role of Akt1 in the advanced cancers and in diabetes-promoted carcinogenesis." Pharmacol Res **145**: 104270.

22. Gao, F., S. Artham, H. Sabbineni, A. Al-Azayzih, X. D. Peng, N. Hay, R. H. Adams, T. V. Byzova and P. R. Somanath (2016). "Akt1 promotes stimuli-induced endothelial-barrier protection through FoxO-mediated tight-junction protein turnover." Cell Mol Life Sci **73**(20): 3917-3933.

23. Sabbineni, H., A. Verma, S. Artham, D. Anderson, O. Amaka, F. Liu, S. P. Narayanan and P. R. Somanath (2019). "Pharmacological inhibition of beta-catenin prevents EndMT in vitro and vascular remodeling in vivo resulting from endothelial Akt1 suppression." Biochem Pharmacol **164**: 205-215.

24. Fanelli, V., Y. Morita, P. Cappello, M. Ghazarian, B. Sugumar, L. Delsedime, J. Batt, V. M. Ranieri, H. Zhang and A. S. Slutsky (2016). "Neuromuscular Blocking Agent Cisatracurium Attenuates Lung Injury by Inhibition of Nicotinic Acetylcholine Receptor-alpha1." Anesthesiology **124**(1): 132-140.

25. Al-Azayzih, A., Missaoui, W. N., Cummings, B. S., & Somanath, P. R. (2016). Liposome-mediated delivery of the p21 activated kinase-1 (PAK-1) inhibitor IPA-3 limits prostate tumor growth in vivo. *Nanomedicine, 12*(5), 1231-1239. doi:10.1016/j.nano.2016.01.003

26. Sabbineni, H., A. Verma and P. R. Somanath (2018). "Isoform-specific effects of transforming growth factor beta on endothelial-to-mesenchymal transition." J Cell Physiol **233**(11): 8418-8428.

27. Goc, A., A. Al-Azayzih, M. Abdalla, B. Al-Husein, S. Kavuri, J. Lee, K. Moses and P. R. Somanath (2013). "P21 activated kinase-1 (Pak1) promotes prostate tumor growth and microinvasion via inhibition of transforming growth factor beta expression and enhanced matrix metalloproteinase 9 secretion." J Biol Chem **288**(5): 3025-3035.

28. Dejana, E., F. Orsenigo and M. G. Lampugnani (2008). "The role of adherens junctions and VE-cadherin in the control of vascular permeability." J Cell Sci **121**(Pt 13): 2115-2122.

29. McKeown, S., A. G. Richter, C. O'Kane, D. F. McAuley and D. R. Thickett (2009). "MMP expression and abnormal lung permeability are important determinants of outcome in IPF." Eur Respir J **33**(1): 77-84.

30. Ito, J. T., J. D. Lourenco, R. F. Righetti, I. Tiberio, C. M. Prado and F. Lopes (2019). "Extracellular Matrix Component Remodeling in Respiratory Diseases: What Has Been Found in Clinical and Experimental Studies?" Cells **8**(4).

31. Vandenbroucke, R. E., E. Dejonckheere and C. Libert (2011). "A therapeutic role for matrix metalloproteinase inhibitors in lung diseases?" Eur Respir J **38**(5): 1200-1214.

32. Yabasin, I. B., J. G. P. Sanches, M. M. Ibrahim, J. Huidan, W. Williams, Z. L. Lu and Q. Wen (2018). "Cisatracurium Retards Cell Migration and Invasion Upon Upregulation of p53 and Inhibits the Aggressiveness of Colorectal Cancer." Front Physiol **9**: 941.

33. Englert JA, Bobba C, Baron RM. Integrating molecular pathogenesis and clinical translation in sepsis-induced acute respiratory distress syndrome [published online ahead of print, 2019 Jan 24]. *JCI Insight*. 2019;4(2):e124061.

34. Lee HS, Moon C, Lee HW, Park EM, Cho MS, Kang JL. Src tyrosine kinases mediate activations of NF-kappaB and integrin signal during lipopolysaccharide-induced acute lung injury. *J Immunol*. 2007;179(10):7001-7011.

35. Taniguchi, K., Karin, M. NF-κB, inflammation, immunity and cancer: coming of age. *Nat Rev Immunol* **18,** 309–324 (2018).

Figure.1

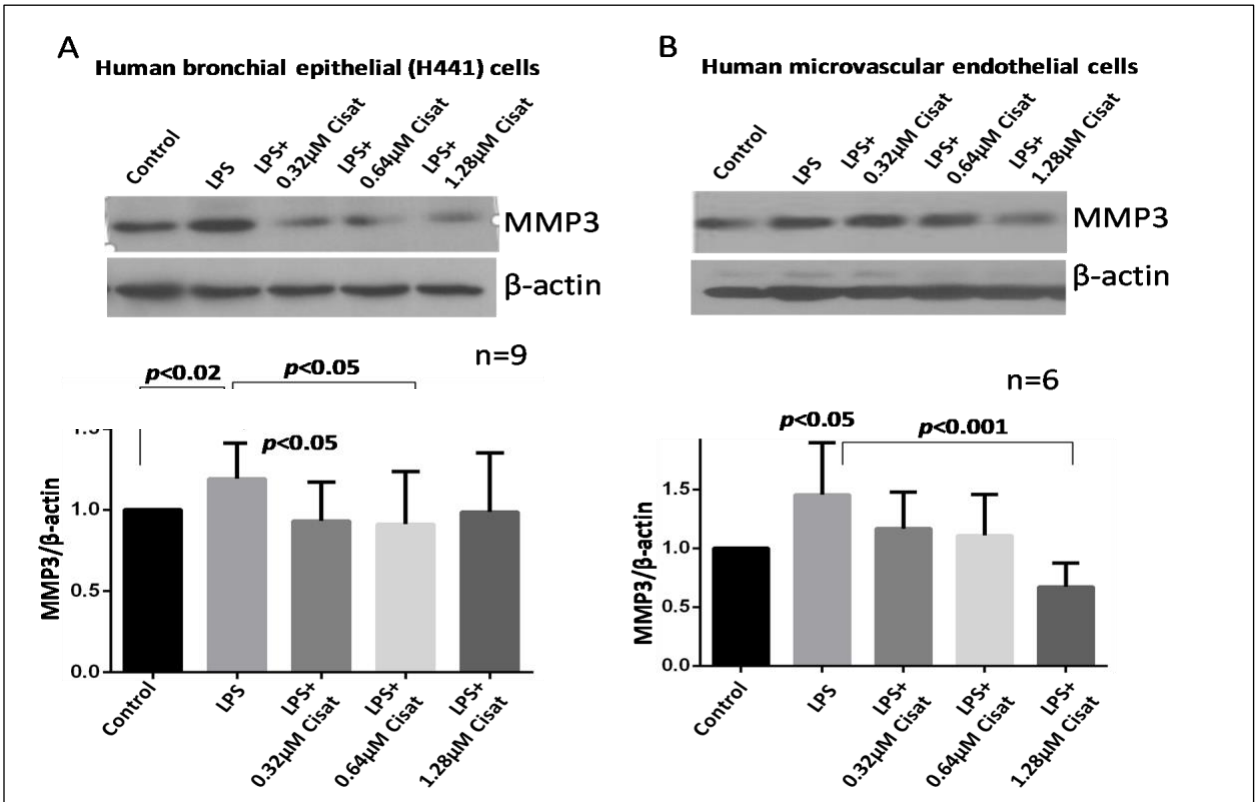

Figure 1: Cisatracurium inhibited LPS-induced increase in MMP3 expression and activity. (A) Representative Western blot images and bar graph with band densitometry analysis indicating increased MMP3 expression in H441 cells with LPS treatment and its reversal by co-treatment with cisatracurium after 24 hours of incubation. **(B)** Representative Western blot images and bar graph with band densitometry analysis indicating increased MMP3 expression in HMECs with LPS treatment and its reversal by co-treatment with cisatracurium after 24 hours of incubation. Data are shown as Mean + SD.

Figure.2

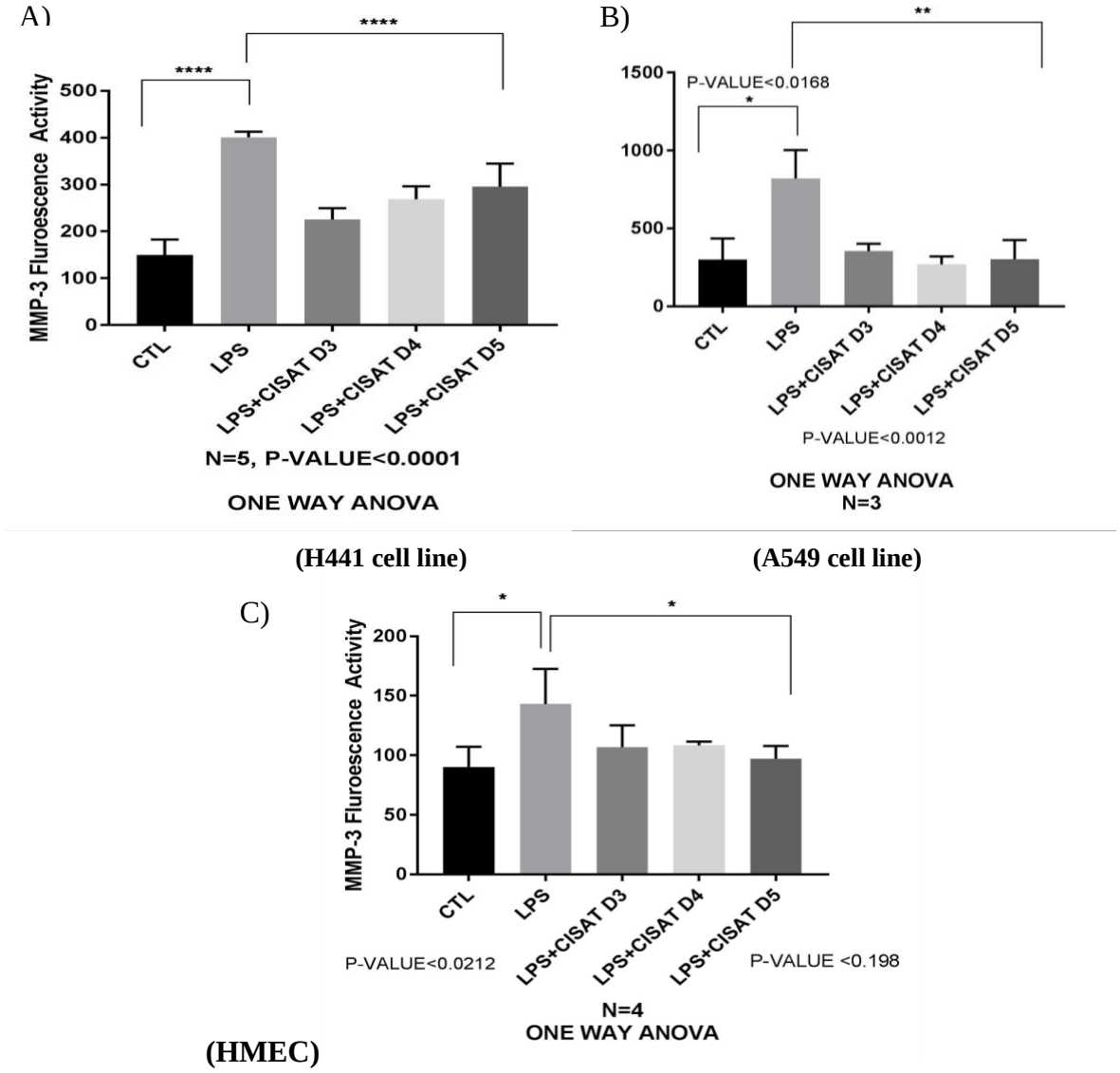

Figure 2: Cisatracurium inhibited LPS-induced increase in MMP3 activity in Microvascular Endothelial (HMEC), Bronchial epithelial (H441) and the alveolar epithelial (A549) cells. Bar graph showing increased MMP3 activity in **A)** H441, **B)** A549 and **C)** HMEC cells by LPS treatment and its significant reversal by co-treatment with cisatracurium for 24 hours. Data are shown as Mean + SD.

D 1:0.03µM**,** D 2: 0.06µM**,** D 3: 0.32µM, D 4:0.64µM**,** D 5:1.28 µM

Figure.3

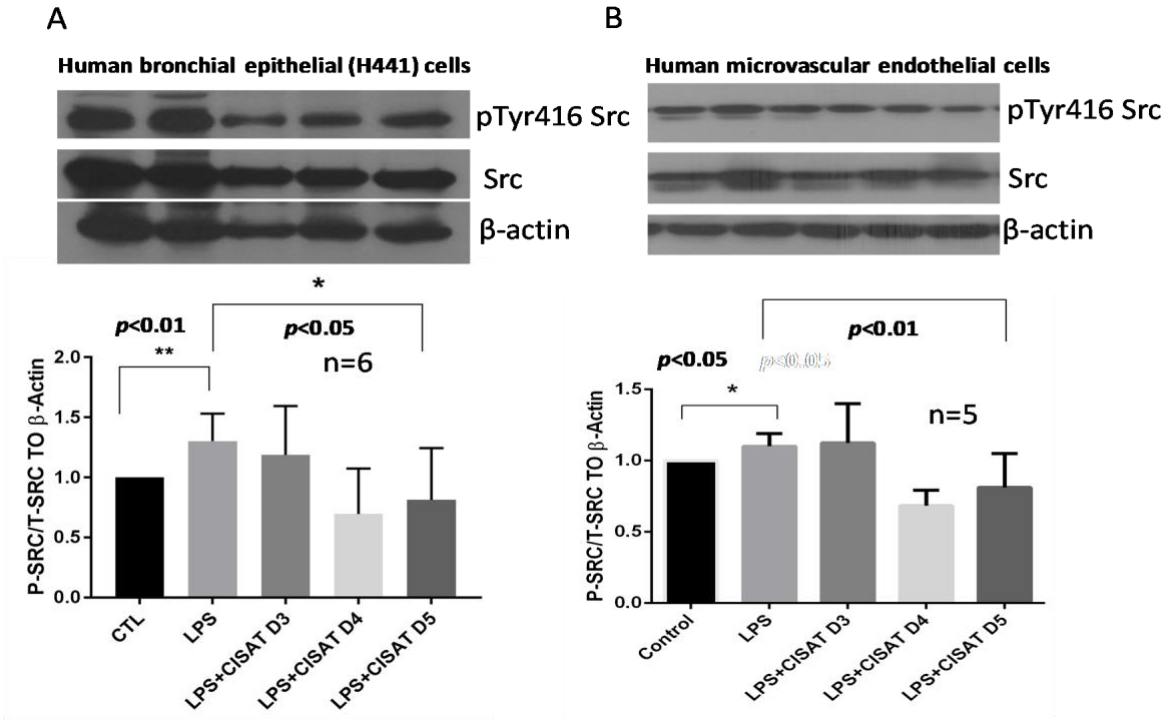

Figure 3: Cisatracurium inhibited LPS-induced Src activity in HMECs and H441 cells. (A) Representative Western blot images and bar graph with band densitometry analysis indicating increased P-SRC/T-SRC expression ratio in H441 cells after 24 hours treatment with LPS and its reversal upon co-treatment with cisatracurium **(B)** Representative Western blot images and bar graph with band densitometry analysis indicating increased P-SRC/T-SRC expression ratio in HMECs after 24 hours treatment with LPS and its reversal upon co-treatment with cisatracurium. Data are shown as Mean + SD.

D1:0.03μM, D 2: 0.06μM, D 3: 0.32μM, D 4:0.64μM, D 5:1.28 μM

Figure.4

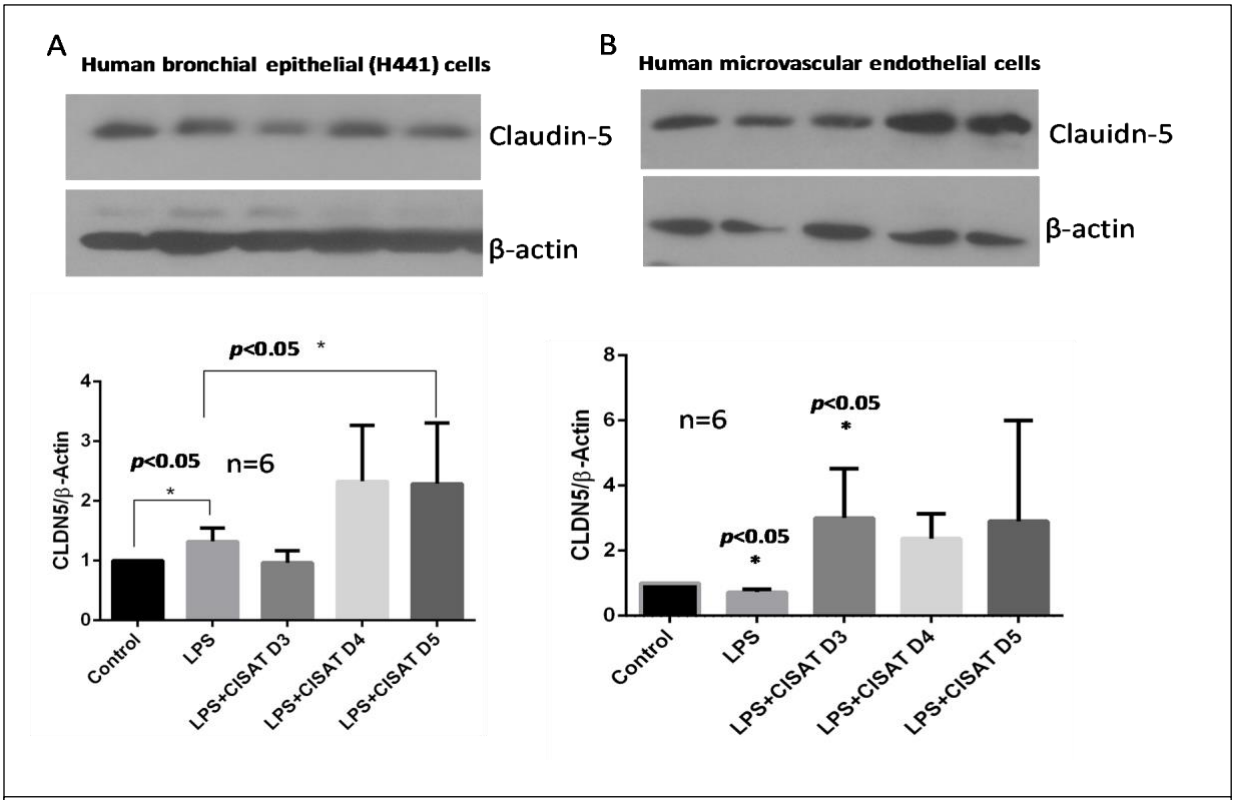

Figure 4: Cisatracurium treatment increased claudin-5 expression in HMECs and H441 cells. (A) Representative Western blot images and bar graph with band densitometry analysis indicating increased claudin-5 expression in H441 cells after 24 hours treatment with LPS and its further increase upon co-treatment with cisatracurium **(B)** Representative Western blot images and bar graph with band densitometry analysis indicating decreased claudin-5 expression ratio in HMECs after 24 hours treatment with LPS and its reversal upon co-treatment with cisatracurium. Data are shown as Mean + SD.

D1:0.03μM, D 2: 0.06μM, D 3: 0.32μM, D 4:0.64μM, D 5:1.28 μM

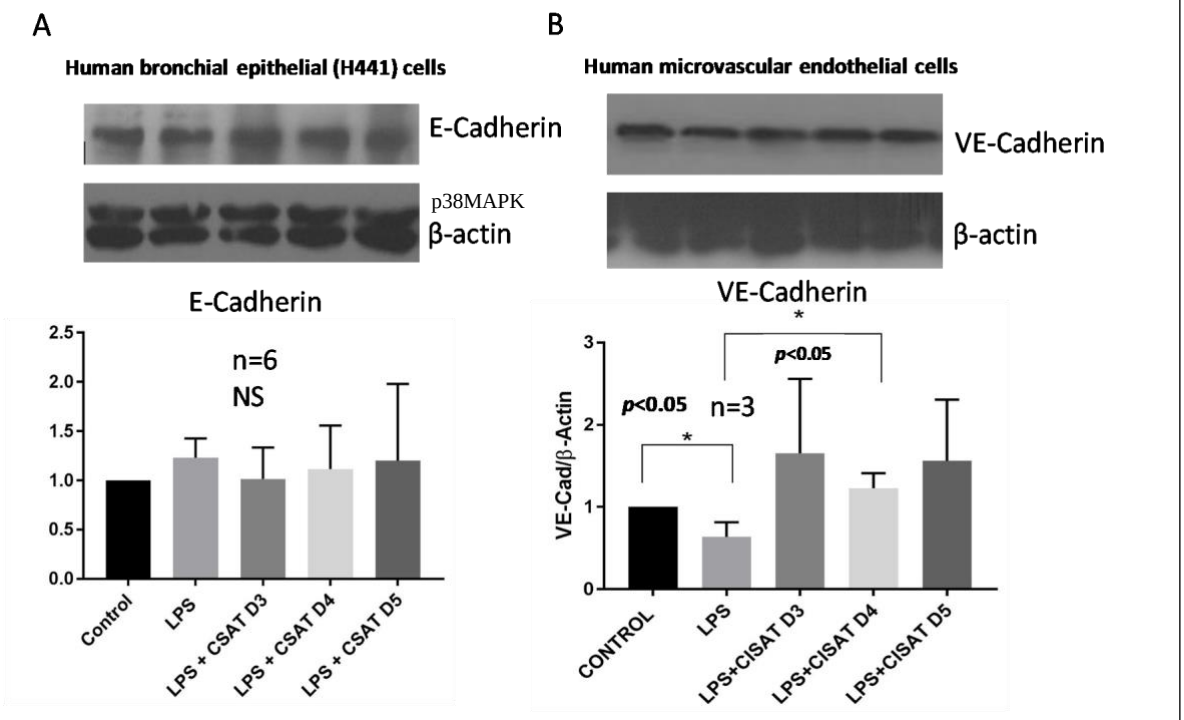

Figure 5: Cisatracurium protected LPS-induced VE-Cadherin loss in HMECs but not E-Cadherins expression in H441 cells. (A) Representative Western blot images and bar graph with band densitometry analysis indicating no change in E-cadherin expression in H441 cells after 24 hours treatment with LPS and/or with cisatracurium **(B)** Representative Western blot images and bar graph with band densitometry analysis indicating decreased VE-cadherin expression in HMECs after 24 hours treatment with LPS and its reversal upon co-treatment with cisatracurium. Data are shown as Mean + SD.

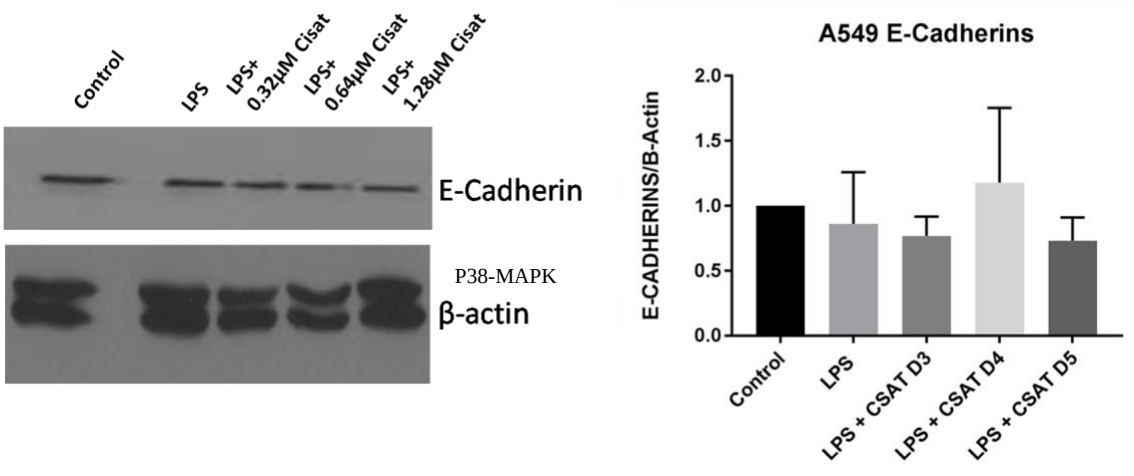

Figure 6: Cisatracurium had no effect on E-Cadherin expression in A549 cells. (A) Representative Western blot images and bar graph with band densitometry analysis indicating no change in E-cadherin expression in A549 cells after 24 hours treatment with LPS and/or with cisatracurium. Data are shown as Mean + SD.

Figure.7

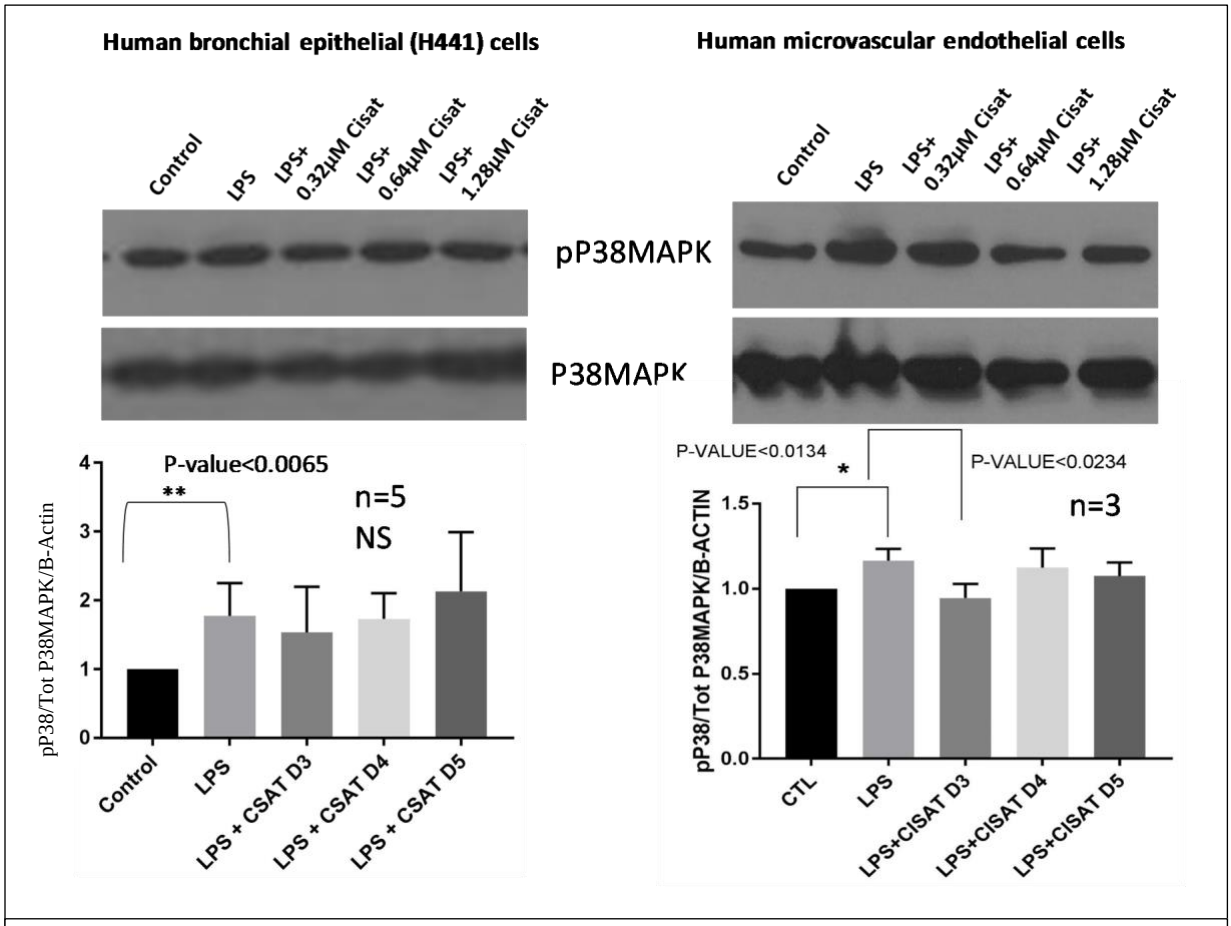

Figure 7: Cisatracurium had no effect on P38MAPK activation in H441 cells and HMECs. (A-B) Western blot images and bar graph with band densitometry analysis indicating no change in pP38MAPK expression in H441 cells HMECs after 24 hours treatment with LPS and/or with cisatracurium. Data are shown as Mean + SD.

D1:0.03µM, D 2: 0.06µM, D 3: 0.32µM, D 4:0.64µM, D 5:1.28 µM

Figure.8

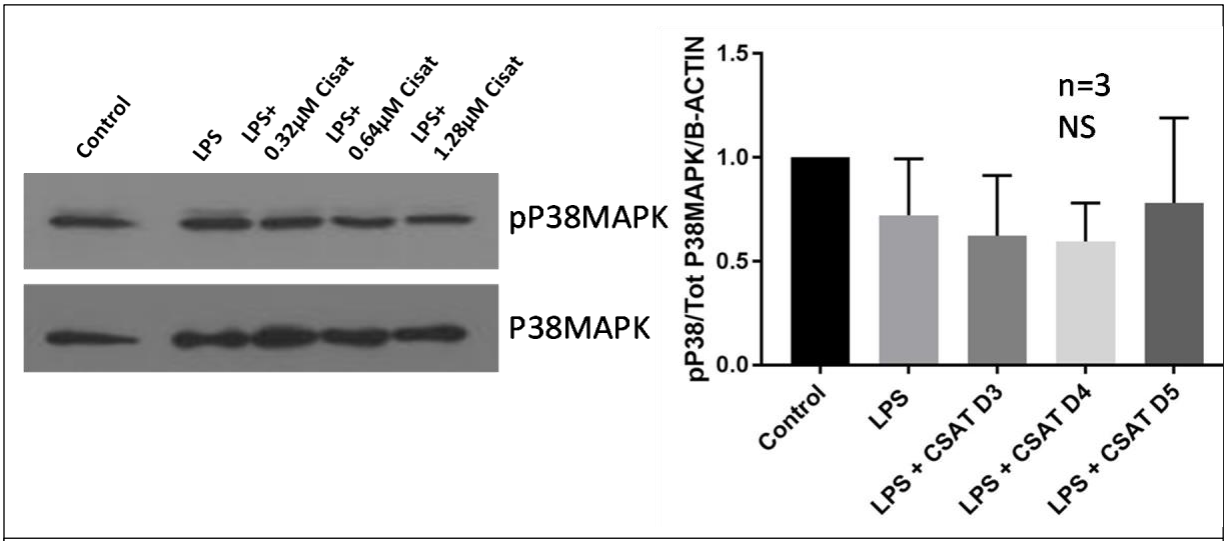

Figure 8: Cisatracurium had no effect on P38MAPK activation in A549 cells. Western blot images and bar graph with band densitometry analysis indicating no change in pP38MAPK expression in A549 cells HMECs after 24 hours treatment with LPS and/or with cisatracurium. Data are shown as Mean + SD.

Figure.9

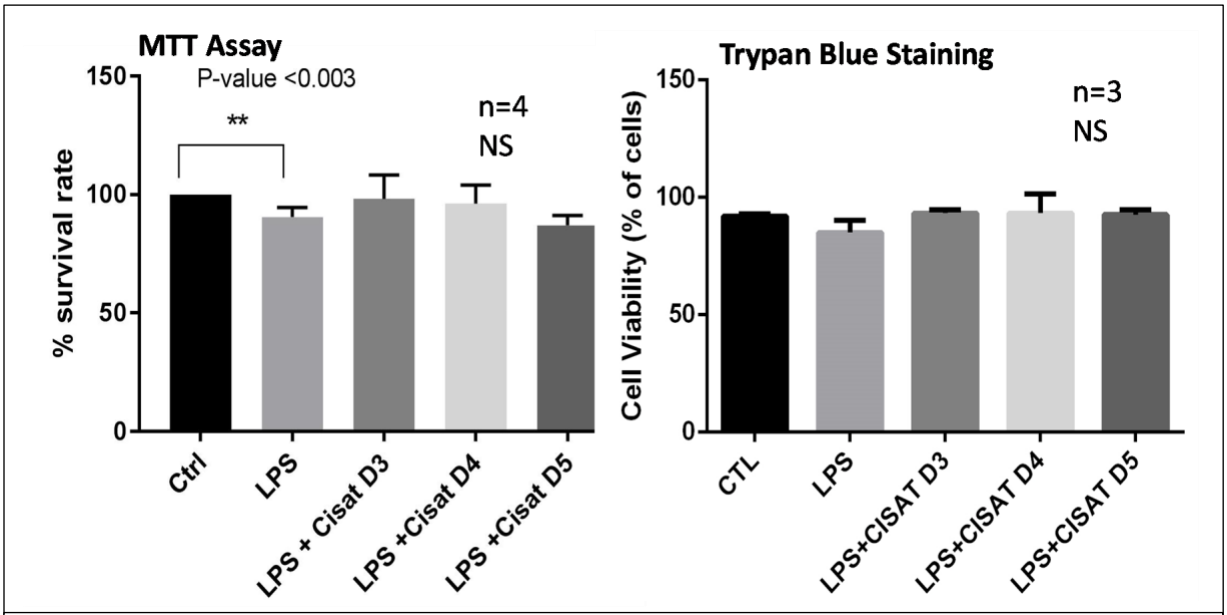

Figure 9: Cisatracurium had no effect on the viability of H441 cells. Bar graph showing a modest decrease in H441 cell survival upon treatment with LPS for 24 hours as evidenced by MMT assay (left) and Trypan blue staining (right) but no change in cell viability by co-treatment with cisatracurium. Data are shown as Mean + SD.

D1:0.03μM, D 2: 0.06μM, D 3: 0.32μM, D 4:0.64μM, D 5:1.28 μM