The Role of Neutrophil Extracellular Traps in Lung Transplantation

By

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Institute of Medical Sciences
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2018

Abstract

Neutrophil extracellular traps (NETs) are a product of the innate immune response. This thesis hypothesized that NETs are produced in response to various forms of lung transplant-related injury, and that the concentration of NETs in the lung predicts lung function. NETs were measured in the lungs and in EVLP perfusate in various models of porcine lung transplant-related injury and ex vivo lung perfusion (EVLP), as well as in clinical EVLP and recipient bronchial wash samples.

NETs were detected in porcine models of lung injury. NET concentration in porcine EVLP perfusate increased over time and varied according to lung injury. In clinical perfusate, NETs were significantly correlated with recipient days on the ventilator and length of stay in the ICU. This thesis demonstrates that NETs are present in lungs at various stages throughout the transplantation process, and that NETs in donor lungs could be used for predicting recipient outcomes.
Acknowledgements

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<th>Description</th>
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<tbody>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
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<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
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<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BW</td>
<td>Bronchial wash</td>
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<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<tr>
<td>DBD</td>
<td>Donation after brain death</td>
</tr>
<tr>
<td>DCD</td>
<td>Donation after cardiac death</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECMO</td>
<td>Extracorporeal membrane oxygenation</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EVLP</td>
<td>Ex vivo lung perfusion</td>
</tr>
<tr>
<td>FiO$_2$</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-Met-Leu-Phe</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILD</td>
<td>Interstitial lung disease</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NE-DNA</td>
<td>Neutrophil elastase-deoxyribonucleic acid</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NOX</td>
<td>Nicotinamide adenine dinucleotide phosphate oxidase</td>
</tr>
<tr>
<td>PaO$_2$</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PGD</td>
<td>Primary graft dysfunction</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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Chapter 1: Introduction
Overview

Lung transplantation is crucial for patients suffering from end-stage lung disease, and the procedure can save the lives of these patients. However, despite vast advancements in the field in recent decades, many obstacles to successful lung transplantation remain. High demand for lung transplant outweighs the number of donor lungs available, resulting in long waitlist times. Furthermore, patients that do receive a lung transplantation can develop primary graft dysfunction, which in turn can manifest in adverse and potentially fatal short- and long-term outcomes.

In order to further improve the outcomes of lung transplantation, a more complete understanding of the cellular processes occurring pre-, peri-, and post-operatively is required. Ex vivo lung perfusion (EVLP) is used clinically to assess donor lung viability prior to transplantation; however, it provides a unique window to assess the donor lung on the macro as well as micro level. Using EVLP as a platform for research allows for the assessment of cellular markers specific to the donor lung.

One cellular marker that has recently been shown to be relevant to lung transplantation is neutrophil extracellular traps (NETs). NETs are the product of an innate immune response, and have been implicated in a variety of lung injury states; in fact, several of the same pathways which result in NET production occur during lung transplantation. Exploring the role of NETs in the lung transplantation process, including the assessment of NETs in ex vivo lung perfusate, may provide insight that is critical to improving lung transplantation outcomes.

1.1 Current State of Clinical Lung Transplantation

Since the first successful lung transplantation performed in 1983 by Dr. Joel Cooper in Toronto, the field of lung transplantation has rapidly evolved with respect to surgical technique, donor lung preservation strategies, and recipient peri-operative management. Lung transplantation is required for survival for end-stage lung disease. The most common indications for lung transplantation are chronic obstructive pulmonary disease (COPD) which constituted about 31% of recipient diagnoses in 2016, interstitial lung disease (ILD; approximately 30%) and bronchiectasis including cystic fibrosis (CF; approximately 16%) (Yusen, Edwards et al. 2016).
Other indications for transplantation include lung failure due to alpha-1 antitrypsin deficiency, re-transplantation, bronchiectasis, sarcoidosis, and pulmonary arterial hypertension.

A severe shortage of donor lungs, in comparison to the number of end-stage lung disease patients requiring life-saving lung transplantation, causes an imbalance in the supply and demand of transplantable lungs. This imbalance manifests as waitlist mortality of approximately 27% (Canadian Institute for Health Information 2017) and a critical need to increase the pool of donor lungs available for transplantation.

Lung transplantation can comprise of either a single lung or bilateral lung transplant, depending on what is required by the recipient. Bilateral transplants are common, constituting about 75% of the transplants performed in 2016 (Yusen, Edwards et al. 2016). In bilateral lung transplantation, each native recipient lung is removed and replaced by the donor lung in a sequential manner. The more severely injured lung is removed first and the graft is implanted, perfused, and ventilated before the second lung is transplanted (Yeung and Keshavjee 2014). In this way, the recipient is continuously supported by at least one lung throughout the procedure. In single lung transplantation, the diseased lung is removed and replaced by the donor lung while the contralateral recipient native lung remains in place. In a single lung transplant, the surgeon opens the chest using an anterolateral or posterolateral thoracotomy, while bilateral transplants can be performed via two anterolateral thoracotomies with or without cutting the sternum (a clamshell incision) (Yeung and Keshavjee 2014). Lungs can also be transplanted as a part of the heart-lung block, if transplantation of both the heart and lungs is required. In a heart-lung transplantation, a single tracheal anastomosis is used, as compared to the two bronchial anastomoses required for the bilateral and single lung transplantations.

Although recent scientific and medical advances have helped improve outcomes post-transplant, the median survival time for adult lung transplant recipients overall is 5.8 years; the unadjusted survival rate drops from 80% at 1 year post-transplant to 32% at 10 years post-transplant (Yusen, Edwards et al. 2016). One major contributing factor to transplant recipient mortality is primary graft dysfunction (PGD). PGD is characterized using the ratio of partial pressure of oxygen (PaO\textsubscript{2}) to fraction of inspired oxygen (FiO\textsubscript{2}). PGD is defined as a PaO\textsubscript{2}/FiO\textsubscript{2} ratio of less than 300 and pulmonary infiltrates visible via radiography within 72 hours post-reperfusion, and affects approximately 30% of lung transplant recipients (Diamond, Arcasoy et al. 2017). PGD is associated with more recipient days on the ventilator, in the intensive care unit
(ICU), and in the hospital. PGD is also associated with greater short- and long-term mortality post-transplant (Christie, Kotloff et al. 2005). A major contributing factor to PGD development is ischemia-reperfusion injury. Ischemia and subsequent reperfusion of lungs is unavoidable in the process of transplantation.

Acute and chronic rejection of the transplanted lung remains a significant challenge in lung transplantation. Almost 30% of transplant recipients experience a rejection episode requiring treatment within the first year after transplant (Yusen, Edwards et al. 2016). Adverse recipient outcomes which occur immediately after transplant, such as the development of PGD (discussed in more depth presently) can contribute to development of worse long-term outcomes. For instance, PGD can lead to development of bronchiolitis obliterans syndrome (BOS), which is the leading cause of death in recipients after the first year post-transplant (Yusen, Edwards et al. 2016) and a significant indicator of rejection. BOS is irreversible, and patients who develop BOS may require a second transplant when associated with severe graft dysfunction. Long-term morbidity rates after transplantation remain high, with approximately 50% of recipients experiencing systemic hypertension within one year post-transplant and 80% within five years (Yusen, Edwards et al. 2016).

**Donor Lung Injury**

Donor lungs can be obtained from living donors as a lobar transplantation, or from deceased donors. There are two major categories of deceased donor lungs: lungs donated after brain death (DBD) and lungs donated after cardiac death (DCD). Each category of lung donor presents its own set of advantages and challenges for successful transplantation. In North America, donor lungs are conventionally obtained from brain-death donors (donation after brain death, DBD). DBD have traditionally been considered more suitable for transplantation due to the uncontrolled “hands-off” period associated with DCD lungs. However, DCD lung recipients have been shown to have similar short- and long-term outcomes to DBD lung recipients (Machuca, Mercier et al. 2015). Living-donor lobar lung transplantation, although developed in the United States, is now largely practiced in Japan with some reports coming from other centers around the world. Although living-donor lobar transplantation has been reported to result in similar outcomes as DBD transplantation (Date, Sato et al. 2015), the procedure carries risk for the donor. Each type
of deceased donor lung has specific potential lung insults as a result of the type of death; injuries that occur in the donor lung can affect the recipient lung function post-transplant (Fisher, Donnelly et al. 2001).

**Donation after brain death (DBD)**

Brain death is defined as the irreversible loss of neurological function. Most DBD organs are made available after donor anoxia, cerebrovascular accident, or head trauma (based on Organ Procurement and Transplant Network Data, as of January 2018). Although widely considered more suitable for transplant than DCD organs, the pathways that are incited in response to brain death lead to systemic hypertension followed by hypotension, anaerobic metabolism, and massive systemic inflammation and upregulation of pro-inflammatory cytokines (Avlonitis, Fisher et al. 2003). The initial brain stem injury induces a sharp increase in systemic norepinephrine and epinephrine, causing hypertension and tachycardia, followed by systemic vasodilation and hypotension (Avlonitis, Fisher et al. 2003). The autonomic nervous system responds to high intracranial pressure by releasing catecholamines in order to increase the mean arterial pressure; this results in vasoconstriction throughout the body and tissue hypoperfusion (Watts, Thom et al. 2013).

The process of brain death is associated with upregulation of pro-inflammatory cytokine release. Brain death is associated with increased serum levels of macrophage-associated cytokines IL-1β and tumor necrosis factor (TNF)-α, as well as neutrophil markers CD18 and CD11b in the blood in a rat model (Avlonitis, Wigfield et al. 2005). Rats who underwent brain death demonstrated increased levels of IL-1, IL-6 and TNF-α, as well as IL-2 and interferon-γ, systemically and in peripheral organs including the lung (Takada, Nadeau et al. 1998). Endothelin-1 is a vasoconstrictor that is upregulated in brain death (Sutherland, Ware et al. 2007). Endothelin-1 is found in alveolar macrophages after brain death and is related to increased expression of MMP-2 and -9 (Sutherland, Ware et al. 2007). IL-8 is a potent neutrophil chemoattractant, and is important in the recruitment and activation of neutrophils at the site of injury. IL-8 is produced during animal models of brain death (Valenza, Coppola et al. 2014), and was found to originate from the alveolar macrophages and epithelial cells in DBD donor lung tissue (Fisher, Donnelly et al. 2001). IL-8 is found in clinical DBD donor lungs, and is associated with worse graft function and mortality in the recipient post-transplant (Fisher, Donnelly et al. 2001). The upregulation of
inflammatory cytokines as a result of brain death can contribute to donor lung injury, even before lung retrieval.

The manifestations of the donor response to brain death are detailed in a rat study of lung transplantation, wherein lungs were retrieved either 15 minutes or 5 hours after brain death, and were compared to lungs from sham donors who did not suffer brain stem injury and death. This study compared the early hemodynamic effects of brain death on reperfusion injury as well as the effects of systemic inflammation in the donor. Specifically, pulmonary vascular resistance (PVR), lung water index, peak airway pressure and graft oxygenation were significantly worse in the 15 minute brain death group than the sham group, up to two hours post-reperfusion (Avlonitis, Wigfield et al. 2007). The recipients of brain death lungs developed elevated inflammatory responses (cytokines and neutrophils) in the lung, suggesting brain death is associated with increased ischemia-reperfusion injury. Interestingly, the lungs retrieved 5 hours after brain death demonstrated significantly lower PVR compared to the 15 minute group, indicating that donors may recover from brain death over time; the authors note that this is consistent with clinical findings (Straznicka, Follette et al. 2002).

Donation after cardiac death (DCD)

The definition of “donation after cardiac death” has been discussed and refined over the past few decades, with alternate descriptive terms including “donation after circulatory death”, and “non-heart beating donation”; however, donation after cardiac death has been the most widely adopted term to describe the donation of organs after cessation of circulation (Thuong, Ruiz et al. 2016). DCD can occur in a controlled or uncontrolled context. The different scenarios for DCD have been categorized into the Maastricht categories of organ donation. Maastricht Category I donors are donors that are dead upon hospital arrival, and so the amount of warm ischemic time is unknown. Category II includes patients who are unsuccessfully resuscitated at the hospital after attempts made by the emergency medical services. Category III donors are awaiting cardiac death following planned withdrawal of life-sustaining therapies. Category IV donors are those who suffer from cardiac arrest after declaration of brain death. Categories II and III protocols often include a “hands-off” period to ensure cessation of circulation prior to preparation for organ retrieval (Thuong, Ruiz et al. 2016).
DCD transplantation is becoming more widely accepted as a way to expand and maximize the donor pool, and multiple centers have reported no adverse short- or long-term outcomes in DCD recipients (Machuca, Mercier et al. 2015, Costa, Shah et al. 2018).

**Acute lung injury**

Acute lung injury (ALI) is defined by the American European Consensus as acute onset of hypoxemia (PaO$_2$/FiO$_2$ less than 300 mmHg) and diffuse, bilateral radiographic infiltrates without evidence left ventricular failure or increased left arterial pressure (Ragaller and Richter 2010). Severely hypoxemic ALI patients can be considered to have acute respiratory distress syndrome (ARDS), if the PaO$_2$/FiO$_2$ is less than 200 mmHg. The mortality rate for patients with ALI is high, at 35-40%, and epidemiological evidence reports 190,000 cases of ALI per year in the United States (Ragaller and Richter 2010).

ALI can result from a variety of insults, both systemic as well as directly targeting the lungs: trauma, blood transfusions, pneumonia, aspiration, mechanical lung ventilation, and ischemia-reperfusion can all contribute to the development of ALI (Folkesson, Matthay et al. 1995, Narasaraju, Yang et al. 2011, Caudrillier, Kessenbrock et al. 2012, Abrams, Zhang et al. 2013, Carrasco Loza, Villamizar Rodriguez et al. 2015, Laubach and Sharma 2016). The characteristics of ALI include epithelial and endothelial cell damage, which contribute increased capillary permeability, leukocyte recruitment, and accumulation of alveolar edema. Epithelial damage and changes in levels of surfactant proteins-A, -B, and –D contribute to diminished pulmonary compliance; reduced compliance is implicated in ALI (Greene, Wright et al. 1999, Johnson and Matthay 2010). Treatments for ALI largely depend on the treatment of the underlying pathology; however, development of ventilatory management and strategies for patients with ALI has been associated with the most significant improvements (Johnson and Matthay 2010, Ragaller and Richter 2010).

**Neutrophils in acute lung injury**

Neutrophils have been described as having a significant role in ALI. IL-8 can recruit neutrophils to the site of injury via chemoattraction. Cellular adhesion molecules, such as selectins,
are expressed on endothelial cells during injury due to upregulated expression of IL-1, TNFα, and complement component 5a. Neutrophils and leukocytes adhere to the endothelium by expressing carbohydrate ligands which bind to the endothelial selectins; this process is called rolling. Leukocyte integrin molecules bind tightly to complementary endothelial molecules in a phase of neutrophil recruitment called adhesion. Integrins can be activated by endothelial-secreted chemokine (CXC)-motif ligand 1 and 8. Finally, leukocytes can undergo diapedesis and transmigrate from the bloodstream through gaps in the endothelial cells into the tissue.

Neutrophils contain several types of granules, which release different products at different stages of neutrophil activation. Secretory vesicles, which are the first subset of granule to be released upon initial endothelial contact (Grommes and Soehnlein 2011) contain the required membrane-associated receptors to initiate the first phase of neutrophil migration after initial inflammatory stimuli signaling.

Tertiary (also known as gelatinase) granules contain some of the same proteins as secondary granules, such as lysozyme and gelatinase, but differ in that tertiary granules are much smaller and lack most of the antimicrobial-specific proteins of the secondary granules (Faurschou and Borregaard 2003). Tertiary granules are released during trans-endothelial migration of the neutrophil into the lung tissue (Grommes and Soehnlein 2011).

Once in the tissue, neutrophils can release their remaining granule contents (Grommes and Soehnlein 2011). Primary (azurophilic) granules contain myeloperoxidase, alpha-defensins, neutrophil elastase, cathepsin G, proteinase 3, and bactericidal/permeability-increasing protein (Faurschou and Borregaard 2003). Myeloperoxidase converts H₂O₂ to HOCl, increase toxicity and microbicidal potential; defensins create transmembrane pores through which they can act antimicrobially; neutrophil elastase, cathepsin G, and proteinase 3 are proteolytic and can induce immune cell activation; and bactericidal/permeability-increasing protein can kill Gram-negative bacteria (Faurschou and Borregaard 2003). Secondary granules, or specific granules, also contain anti-microbial agents, such as lactoferrin, lysozyme, and hCAP18 which becomes the antimicrobial polypeptide LL-37 upon activation by proteinase 3, as well as matrix metalloproteinases-8, -9, and -25, which can disrupt and digest structural components of the extracellular matrix. The release of azurophilic and specific granules, therefore, is a potent antimicrobial event.
Neutrophil movement from the capillaries to alveoli involves a change in neutrophil morphology, as the neutrophils have to adjust to the size of the pulmonary capillaries which can be as small as one third of the diameter of the neutrophil (Grommes and Soehnlein 2011). The reduced deformability of neutrophils in systemic inflammatory response syndrome (SIRS)-related ALI and other injurious settings means that neutrophils can no longer pass easily through the microvasculature; their increased rigidity causes them to accumulate in the capillaries, where they release cytotoxic granular contents, reactive oxygen species, and contribute to endothelial damage. Attenuation of neutrophil rigidness improves ALI parameters, such as increased oxygenation, decreased pulmonary neutrophil elastase content, and lung injury score (Inoue, Tanaka et al. 2006). Other forms of lung injury, including ventilator-induced lung injury, acid aspiration, and ischemia-reperfusion injury, also recruit neutrophils to the pulmonary microvasculature, where they are sequestered and induce local injury to the lung endothelium and epithelium (Folkesson, Matthay et al. 1995, Ross, Tribble et al. 1999, Kim, Chung et al. 2014). The injury to pulmonary endothelium and epithelium, as well as remodeling of endothelial tight junctions, causes increased permeability of the alveolar-capillary barrier, allowing for edema and the “hallmark” feature of neutrophil emigration and activation into the airspaces of the lung (Grommes and Soehnlein 2011).

As a part of the innate immune defense system, neutrophils are potent antimicrobials. The production and release of oxidants and reactive oxygen species (ROS) is a further mechanism by which neutrophils can perform antimicrobial functions; ROS can be released into the phagosome to help degrade pathogens, as well as contribute to intracellular signaling. Myeloperoxidase can catalyze ROS production after inflammatory signaling. A major source of ROS in inflammation, however, is from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase (NOX) homologs can be activated by chemokine signaling in inflammation and contribute to the production of ROS by inducing an oxidative burst. NOX2 in neutrophils is thought to be a mediator of NOX-dependent NETosis, as inhibition of NOX2 attenuates NETosis (Remijsen, Vanden Berghe et al. 2011); however, calcium-ionophore induced NETosis is independent of NOX2 activity (Douda, Khan et al. 2015). Interestingly, NOX4, which is the NOX homolog predominantly found in human lung microvasculature and arterial endothelium, has a potential protective role in the vasculature rather than a destructive role (Mittal, Siddiqui et al. 2014).
Primary Graft Dysfunction

PGD is a specific type of ALI which develops in lung transplant recipients within the first 72 hours after reperfusion. PGD is thought to affect between 11-25% of lung transplant recipients (Christie, Carby et al. 2005). PGD is the leading cause of morbidity and mortality post-transplant, and is a major risk factor for adverse long-term outcomes, including bronchiolitis obliterans development and chronic lung allograft dysfunction.

Currently, there is no treatment available specifically targeting PGD; patients are generally provided with supportive therapies such as protective ventilation and extracorporeal membrane oxygenation (ECMO) (Suzuki, Cantu et al. 2013). Prevention and amelioration of ischemia-reperfusion injury, and treatment of subsequent PGD, is the subject of many animal models of lung transplantation (Motoyama, Chen et al. 2014, Sayah, Mallavia et al. 2015, Iskender, Sakamoto et al. 2016, Machuca, Cypel et al. 2017).

PGD can be graded with regard to the severity of the dysfunction, with a PGD of grade 0 being the absence of PGD, and PGD grade 3 being the most severe iteration of the graft dysfunction. Specifically, the ISHLT defined PGD grade 0 as a PaO$_2$/FiO$_2$ ratio greater than 300 and lack of radiographic infiltrates; PGD grade 1 as PaO$_2$/FiO$_2$ ratio less than 300 and the presence of infiltrates; PGD grade 2 as PaO$_2$/FiO$_2$ ratio of 200-300 and the presence of infiltrates; and PGD grade 3 as PaO$_2$/FiO$_2$ ratio less than 200 and the presence of infiltrates. Radiographic infiltrates can include a variety of abnormalities, such as ground-glass opacities, thickening of the bronchi or vasculature, and interstitial and alveolar opacities in the lower and middle lobes (Suzuki, Cantu et al. 2013). PGD can be classified within 6 hours of reperfusion as “time zero” (T0), and at every 24 hours for the first 72 hours (T24, T48, T72) (Christie, Carby et al. 2005). The purpose of grading scheme is to capture the variability of PGD seen in patients after transplantation in a way that is standardized and meaningful for researchers. However, this grading scheme is evolving since its initial conception to better address the needs of the research community (Christie, Keshavjee et al. 2008).

Ischemia-Reperfusion Injury
Ischemia-reperfusion injury is the injury that results from the stopping and subsequent restarting of blood flow to the organ. Ischemia-reperfusion injury has been shown to be bimodal, and short amounts of ischemic time have been shown to have a protective effect against later ischemic incidents (Soncul, Oz et al. 1999). However, with regard to organ transplantation, ischemia and reperfusion often present a challenge due to the resulting injury.

Ischemia-reperfusion injury is the cause of PGD in lung transplant recipients. PGD is a major risk factor for poor long-term outcomes, notably the development of BOS (Huang, Yusen et al. 2008). Therefore, ischemia-reperfusion injury represents not just a source of immediate injury to the recipient, but amelioration of ischemia-reperfusion injury at the time of transplantation may have important impact on later development of BOS.

Ischemia implies lack of oxygen delivered to the tissue, meaning that anaerobic metabolism takes place in the organ. Different organs present different susceptibilities to ischemia-reperfusion injury, and varying severity of resulting injury (Kalogeris, Baines et al. 2012). The lung is unique in that air retained in the alveoli can continue to provide oxygen to the lung, and thus aerobic metabolism can continue; however, the ischemic lung still suffers from detrimental oxidative stress (Ferrari and Andrade 2015).

Ischemia and reperfusion can incite the innate immune response, resulting in inflammation and tissue injury. While this is a form of sterile injury, toll-like receptors (TLRs), which respond to bacterial infection, are also implicated in lung ischemia-reperfusion injury. For instance, a TLR4-knockdown model of ischemia-reperfusion injury resulted in attenuated lung injury as measured by BAL cell counts, myeloperoxidase content in lung tissue, and lung permeability index (Merry, Phelan et al. 2015).

TLR signaling activates the innate and adaptive immune response. In the context of lung transplantation, TLRs are pattern recognition receptors that can be activated by a variety of endogenous molecules as well as lipopolysaccharide. TLRs play specific roles in the context of lung ischemia and reperfusion (Phelan, Merry et al. 2015), and are associated with increased production of cytokines soon after transplant (Andrade, Kaneda et al. 2006); upregulation of pro-inflammatory cytokines, such as IL-6, TNFα, and IL-8, contribute to the injury seen in the lung. Other factors contributing to ischemia-reperfusion injury in the lung include increased pulmonary vascular resistance (PVR) due to increased vasoconstriction resulting from increased endothelins.
and decreased nitric oxide, cell damage and death, and leukocyte activation and infiltration in the lung.

Ischemia-reperfusion injury consists of two phases: the early phase is mediated by donor characteristics, including donor macrophage activation, and the later phase is characterized by recipient neutrophils and lymphocytes (Ferrari and Andrade 2015). Recently, Zheng et al. demonstrated through a murine model that donor non-classical monocytes are mediated by TLRs to produce chemokine (CXC) motif ligand 2 and subsequently mediate neutrophil infiltration and subsequent ischemia-reperfusion injury in the recipient (Zheng, Chiu et al. 2017).

1.2 Ex vivo lung perfusion

One strategy to mitigate the effects of ischemia reperfusion injury in the recipient is through the functional and physiological assessment of donor lungs prior to transplantation. Ex vivo lung perfusion is a rapidly developing field in the realm of lung transplantation, with the aim of expanding the donor pool as well as providing a platform for personalized medicine for the organ, and thus optimizing lung transplant outcomes. Ex vivo lung perfusion (EVLP) utilizes normothermic perfusion of lungs which are retrieved from the donor and perfused in an isolated circuit, so that the pulmonary physiology and function can be assessed. Normothermic ex vivo perfusion preserves the lungs in a physiologic state, reducing the ischemic time and maintaining lung function. EVLP was first applied clinically in 2001 in order to assess the function of bilateral lungs from a DCD donor, of which the right lung was successfully transplanted (Steen, Sjoberg et al. 2001). In 2011, the Toronto group published the results from a landmark clinical trial showing that transplant recipients who received lungs from high-risk donors that demonstrated physiological stability throughout 4 hours of EVLP had similar outcomes to recipients who received lungs selected using standard criteria (Cypel, Yeung et al. 2011). Since that time, the clinical use of EVLP has been implemented in centers around the world.

Clinical use of EVLP

Today, three major protocols for EVLP exist: the Toronto protocol, the Lund protocol, and the Organ Care System protocol. These protocols share the same fundamental perfusion circuit,
consisting of a pump, leukocyte filter, oxygenator membrane with temperature control, and a means of storing the returned perfusate in a reservoir. The lungs are attached to a ventilator by the trachea. The Toronto protocol for EVLP employs a “closed” left atrium (the atrium is cannulated), a slow increase in perfusate flow to 40% of the predicted donor cardiac output, and simultaneous gradual increase in perfusate temperature from 4°C to 37°C, protective ventilation strategies, and acellular perfusate comprised of low-potassium dextrose solution supplemented with human albumin (Steen® Solution; XVIVO Perfusion) (Machuca and Cypel 2014). While the circuit and its components are not changed during EVLP, Steen® can be replaced as necessary to ensure adequate volume in the reservoir. The Lund protocol differs in that the left atrium remains open for effluent drainage, the perfusate is cellular and comprised of red blood cells and Steen® solution, and the target flow is 100% of donor cardiac output; the Organ Care System protocol targets flow at 2.5 liters per minute, uses an open left atrium, and a cellular perfusate (Van Raemdonck, Neyrinck et al. 2015).

EVLP contributes to expanding the donor pool by assessing lungs that are not conventionally accepted for transplant (for example, DCD lungs) on the basis of their physiology and functionality, and thus accepting lungs that would otherwise be rejected. Post-transplant clinical outcomes from lungs reconditioned with EVLP are on par with or superior to lungs preserved under hypothermic static conditions (Cypel, Yeung et al. 2011, Boffini, Ricci et al. 2014). EVLP is currently the clinical standard of care in Toronto, which has led to a 15% increase in the number of lungs available for transplant (Cypel, Yeung et al. 2012). EVLP also provides the opportunity for personalized medicine for the individual organ, where ideally each lung could be specifically treated to optimize its performance before transplantation.

EVLP as a platform for research and experimental therapeutics

EVLP can be used to expand the donor pool and provide accurate assessments of donor lung function. However, it is also essential to note the opportunity of EVLP as a platform for research regarding targeted therapeutics and an understanding of the biological processes in the donor lung on a cellular and molecular level. Since the lungs are in a circuit and thus isolated from any other organs, EVLP can be used to administer high doses of therapeutic agents directly to the lung without risk of systemic side effects. Recent research in the field has highlighted the different
therapies and avenues of administration using EVLP in animal models: beneficial therapies can be delivered intravascularly via the perfusate or into the airways, and include anti-inflammatory agents (Charles, Mehaffey et al. 2017, Lin, Chen et al. 2017), anti-oxidants (Yamada, Iskender et al. 2017), gene therapy (Machuca, Cypel et al. 2017), stem cells (Mordant, Nakajima et al. 2016, Martens, Ordies et al. 2017), and more. Furthermore, EVLP can be used to assess specific cellular biomarkers in the lung by analysis of the perfusate. Detection of biomarkers in the donor lung prior to transplantation that will predict recipient outcomes are crucial to determining the fate of the perfused lung, and this has been an area of active investigation. Relevant biomarkers predicting lung function after transplantation which are detectable in EVLP perfusate to date include the pro-inflammatory interleukin (IL)−1β (Andreasson, Borthwick et al. 2017), epithelial cell death markers M30 and high mobility group box 1 (Hashimoto, Cypel et al. 2017), exhaled carbon monoxide (Maignan, Gennai et al. 2017), soluble adhesion molecules (Hashimoto, Cypel et al. 2017), and endothelin-1 (Machuca, Cypel et al. 2015), among others.

1.3 Neutrophil extracellular traps

Neutrophil extracellular traps (NETs) were first described in the context of neutrophil cell death: the authors demonstrated a change in neutrophil morphology from neutrophils treated with phorbol myristate acetate (PMA) which was vastly different from either apoptosis or necrosis, and included decondensation of chromatin and nuclear membrane breakdown (Takei, Araki et al. 1996). NETs were subsequently described the product of an active process of neutrophil cell death, termed NETosis, whose function served to trap and kill bacteria (Brinkmann, Reichard et al. 2004).

NETs are comprised of extracellular deoxyribonucleic acid (DNA) embedded with histones and neutrophil primary granular proteins such as neutrophil elastase (NE), cathepsin G, and myeloperoxidase (Brinkmann, Reichard et al. 2004). Secondary and tertiary granular proteins have also been described in NETs (Brinkmann, Reichard et al. 2004). The extracellular DNA forms fiber-like structures that are 15-17 nm in diameter (Brinkmann, Reichard et al. 2004). This chromatin is embedded with the NET-related proteins results in a mesh-like structure which, in vitro, float in media “like a spider’s web does in moving air” (Cooper, Palmer et al. 2013).

The physical structure of NETs allows them to perform their cytotoxic functions: the so-called web of extracellular DNA serves to ensnare pathogens and contain them in close proximity.
to the antimicrobial NET proteins. NETosis can be initiated by a wide variety of inflammatory stimuli, from pathogen recognition to neutrophil chemotaxis signaling. As a part of the innate immune response, the functionality of NETs extends beyond targeting bacteria, to trapping and neutralizing a range of pathogens including fungi, such as *Candida albicans* (Urban, Reichard et al. 2006), and viruses such as human immunodeficiency virus-1 (Saitoh, Komano et al. 2012). Although beneficial and effective in these circumstances, NETs have also been implicated in a variety of disease states.

**Overview of NETosis pathways and regulators**

NETosis is a caspase-independent form of neutrophil cell death; in fact, caspase activity is thought to be inhibited during NETosis by ROS production and neutrophil autophagy (Remijsen, Kuijpers et al. 2011). There are different signaling pathways that can lead to NETosis in response to pathogen invasion or inflammation. The first description of NETs’ ability to kill bacteria used lipopolysaccharide, PMA, and IL-8 as inciting stimuli (Brinkmann, Reichard et al. 2004). While PMA is a chemical agent, it is a potent activator of NADPH oxidase, both dependently and independently of the phosphatidylinositide 3-kinase pathway, and has been well described as an inflammatory activator in cells (Karlsson, Nixon et al. 2000). Lipopolysaccharide, or endotoxin, is a component of the outer membrane of Gram-negative bacteria, and induces a strong response from the immune system; in studying NETosis, lipopolysaccharide represents the challenge neutrophils would face from bacteria. IL-8 is a well described inducer of neutrophil chemotaxis, and is active in sterile inflammation such as ischemia-reperfusion injury.

NETs have been shown to exert a cytotoxic effect on endothelial cells, specifically from the exposure of histones to the endothelium (Saffarzadeh, Juenemann et al. 2012). Another study demonstrated that neutrophils and endothelial cells cultured together resulted in endothelial cell death caused by high levels of IL-8 as well as increased NETosis, suggesting that activated endothelium and neutrophils undergoing NETosis may play into a positive feedback loop: endothelial cells can be activated and induce NETosis, which then incites further endothelial injury causing the endothelial to release more pro-inflammatory and pro-NETosis mediators (Gupta, Joshi et al. 2010, Kazzaz, Sule et al. 2016).
Platelets are anucleated megakaryocyte-derived cells that contribute to blood clotting. Platelets have been described in a variety of studies as being important mediators of NETosis. Endothelial activation or vascular injury can cause platelets to aggregate, leading to coagulation and thrombin formation. Platelets are implicated in bacterial-, viral-, and sterile inflammation-induced NETosis (Clark, Ma et al. 2007, Caudrillier, Kessenbrock et al. 2012, Jenne, Wong et al. 2013). The activation of the Raf/MEK/ERK pathway is thought to be necessary for platelet-mediated NETosis, as it is for PMA-induced NETosis (Caudrillier, Kessenbrock et al. 2012); while ERK signaling was found to be significant for NETosis, NADPH oxidase was not found to play a major role in platelet-induced NETosis (Carestia, Kaufman et al. 2016). Platelets stimulated with lipopolysaccharide or Pam3CSK4 (components of Gram-negative and Gram-positive bacterial cell walls, respectively) greatly increased NETosis activity, in comparison to basal levels as well as neutrophil stimulation with only lipopolysaccharide or Pam3CSK4 and without stimulated platelets (Carestia, Kaufman et al. 2016). Carestia et al. also found that NETs were also formed in the presence of platelets stimulated with classical platelet agonists, such as collagen, thrombin, and adenosine diphosphate. The platelet agonist arachidonic acid and leukotriene B4, a product of arachidonic acid metabolism and a neutrophil chemoattractant, were found to be potent inducer of NETs. GPIb, a platelet surface receptor involved in activation, and CD18 on neutrophils were found to be key receptors for platelet-mediated NETosis, while P-selectin, which mediates platelet-neutrophil interactions, was not found to play a significant role. Thromboxane A2 was found to be the upstream molecular mediator of platelet-mediated NETosis, followed further downstream by von Willebrand factor and platelet factor 4 (Carestia, Kaufman et al. 2016).

Although understanding the mechanisms of NETosis is not entirely elucidated, there is some consensus around key steps that have been shown to be essential for the formation of NETs. Essential to NETosis is the decondensation of intracellular chromatin. This is mediated by the posttranslational citrullination of histones, which is a result of the function of the enzyme peptidyl arginine deiminase 4 (Wang, Li et al. 2009). Peptidyl arginine deiminase 4 converts positively charged arginine residues into uncharged citrulline; the hypercitrullination of histones is required for the hallmark rapid chromatin decondensation in NETosis (Wang, Li et al. 2009, Li, Li et al. 2010, Leshner, Wang et al. 2012). Peptidyl arginine deiminase 4 activity is dependent on the presence of calcium ions; peptidyl arginine deiminase 4 inhibition reduces calcium ion- or Gram-negative bacteria-induced NETosis, indicating that histones are regulators in NETosis as well as
antimicrobial agents (Remijsen, Kuijpers et al. 2011). Histone citrullination can be a step in the NETosis pathway, but citrullination of histones is not sufficient for induction of NETosis (Remijsen, Kuijpers et al. 2011) and may not occur depending on the NETosis stimuli – specifically, histone citrullination was not shown in response to PMA-induced NETosis (Kenny, Herzig et al. 2017).

Another common requirement for NETosis is the activation of neutrophil elastase, although calcium ionophore-induced NETosis has been shown to occur when neutrophil elastase is inhibited (Kenny, Herzig et al. 2017). Papayannopoulous et al. demonstrated that activated neutrophil elastase was required and sufficient for the decondensation of nuclear chromatin in vitro, and this function is promoted when neutrophil elastase synergizes with myeloperoxidase (Papayannopoulous, Metzler et al. 2010). This group further demonstrated that active neutrophil elastase is required for NET formation, as neutrophil elastase acts to cleave histones H4 and H2B, and that neutrophil elastase migrates to the nucleus during NETosis while myeloperoxidase remains granular until co-localizing with neutrophil elastase and DNA during the NET release (Papayannopoulous, Metzler et al. 2010). Thus, the granular components of neutrophils act as antimicrobial components of NETs as well as regulators of NET formation.

NETosis can occur in a NADPH oxidase (NOX)-dependent manner as well as a NOX-independent manner. Douda et al. first described the NOX-independent pathway for NETosis as relying on influx of intracellular calcium for peptidyl arginine deiminase 4 activation and subsequent chromatin decondensation. This pathway is mediated by mitochondrial ROS and a small conductance potassium channel member SK3 (Douda, Khan et al. 2015). Calcium ionophore-induced NOX-independent NETosis occurs more rapidly than NOX-dependent NETosis induced by PMA (Douda, Khan et al. 2015). The pathways also differ in the kinases that are activated. While both NOX-dependent and –independent NETosis require activation of protein kinase B, a kinase involved in cell survival, only the NOX-dependent pathway requires activation of ERK (Douda, Khan et al. 2015). The role of transcription in NETosis has recently been shown to be important to both NETosis pathways, differing in the specific kinases and resulting transcription factors that are activated. Both pathways show activation of protein kinase B, p38, and Src, and of transcription factors hypoxia inducible factor 1-alpha, nuclear factor κB, signal transducer and activator of transcription 3, and p53, among others; the NOX-dependent pathway specifically demonstrates upregulation of signal transducer and activator of transcription 1,
forkhead box O3, and estrogen receptor 1, while the NOX-independent pathway shows higher levels of nuclear factor of activated T-cells, and signal transducer and activator of transcription 5, among others (Khan and Palaniyar 2017).

The NOX-dependent pathway is thought to be activated by lipopolysaccharide, PMA, and microbe presentation (Khan and Palaniyar 2017). Key features of the NOX-dependent pathway are thought to include autophagy and ROS generation. Neutrophils treated with PMA demonstrate vacuolization characteristic of autophagy, and inhibition of the phosphatidylinositide 3-kinase pathway, which also inhibits autophagy, prevents neutrophil chromatin decondensation while leaving NOX2 activity intact (Remijsen, Vanden Berghe et al. 2011). Interestingly, inhibition of either NOX2 or autophagy in PMA-treated neutrophils induced caspase activation and led to apoptosis-like membrane blebbing in the neutrophils; this was not observed in control neutrophils treated with PMA (Remijsen, Vanden Berghe et al. 2011). This indicates the importance of autophagy in NOX-dependent NETosis pathways, and demonstrates how NETosis is another form of programmed cell death that differs from apoptosis.

Autophagy, which is an important process for cell homeostasis, has been further implicated in NETosis via regulation by the mammalian target of rapamycin pathway. formyl-Met-Leu-Phe (fMLP) is a peptide in bacteria, which was used to stimulate neutrophils and demonstrate that mammalian target of rapamycin inhibition increased NETosis in fMLP-stimulated neutrophils (Itakura and McCarty 2013). However, these findings contradict earlier observations that rapamycin inhibits formation of NETs from neutrophils stimulated with lipopolysaccharide (McInturff, Cody et al. 2012).

ROS are thought to contribute to NETosis via the release of neutrophil elastase, myeloperoxidase, and subsequent NET release in PMA-induced NETosis, thus acting as a crucial player in NETosis (Papayannopoulos, Metzler et al. 2010). ROS can be produced by activated neutrophils. However, different NETosis stimuli have different ROS requirements: ROS are produced by neutrophils stimulated with PMA, calcium ionophore A23187, C. albicans, and Group B Streptococcus, and ROS are required for NETosis in neutrophils stimulated with PMA and C. albicans (Kenny, Herzig et al. 2017). ROS may promote NETosis by preventing caspase activity directly (Fadeel, Ahlin et al. 1998) or indirectly via activation of nuclear factor κB and subsequent caspase inhibition (Remijsen, Kuijpers et al. 2011). ROS are well-documented effectors of cell and tissue damage, especially in the context of oxidative stress and ischemia-
reperfusion (Ferrari and Andrade 2015); activation of NETosis may be one way that ROS contribute to damage in the context of lung transplant-related injury.

NETosis was initially described as a form of neutrophil cell death; this pathway is called “suicidal NETosis”. Suicidal NETosis is thought to occur over the course of approximately four hours after neutrophil simulation in vitro, and can occur in response to PMA, which activates the NOX-dependent pathway (Douda, Khan et al. 2015), lipopolysaccharide, and other stimuli. In suicidal NETosis, the neutrophil is ultimately lysed, which allows the NET that has formed from de-condensed chromatin and antimicrobial granular components within the cell membrane to be actively extruded into the extracellular space.

Recently, it has been shown that neutrophils can retain their phagocytic capabilities and membrane integrity after the extrusion of NETs, in a process called “vital NETosis” (Yipp and Kubes 2013). In contrast to suicidal NETosis, vital NETosis can occur relatively quickly, within approximately 30 minutes after stimulation. Clark et al. first demonstrated this phenomenon in the context of sepsis, and showed it was largely dependent on platelet TLR4 (Clark, Ma et al. 2007). Importantly, this study showed that cellular membrane of the neutrophils that extruded NETs remained intact. Similarly, Pilsczek et al. described a neutrophil response to Staphylococcus aureus in which NETs were detected within 10-15 minutes of exposure to S. aureus, but neutrophil membranes remained intact (Pilsczek, Salina et al. 2010). Further investigations demonstrated that NETs could be rapidly created and extruded through vesicles in response to microbes such as Candida albicans (Byrd, O'Brien et al. 2013), and that NET-forming neutrophils can retain functional capacity after extrusion of the NETs (Yipp, Petri et al. 2012).

While NETs are the focus of this thesis, it is worth mentioning that extracellular traps (ETs) have been detected from other innate immune cell types, including eosinophils (Yousefi, Gold et al. 2008), monocytes (Granger, Faille et al. 2017), macrophages (Aulik, Hellenbrand et al. 2012), and mast cells (von Kockritz-Blickwede, Goldmann et al. 2008). Eosinophil extracellular traps were first described in the context of gastrointestinal inflammation, wherein eosinophils primed with IL-5 or interferon-γ release mitochondrial DNA rapidly and in a “vital” manner after lipopolysaccharide challenge with retained eosinophil viability after DNA release (Yousefi, Gold et al. 2008). Eosinophil extracellular traps have been implicated in a variety of eosinophil-mediated pathologies, including allergic diseases such as asthma and contact dermatitis, experimental sepsis, and the autoimmune disease bullous pemphigoid (Yousefi, Simon et al. 2012). Mast cell-derived
extracellular traps were the first innate immune cell after neutrophils that were shown to be able to produce extracellular traps. Mast cell-derived extracellular traps are similar to NETs in that they act as an antimicrobial response to bacterial challenge, are comprised of nuclear DNA, histones, and LL-37, and were first described as the product of a ROS-mediated form of cell death (von Kockritz-Blickwede, Goldmann et al. 2008); unlike NETs, myeloperoxidase and neutrophil elastase do not play a significant role in mast cell-derived extracellular traps (Mollerherm, von Kockritz-Blickwede et al. 2016). A scoping review of the literature macrophage-derived extracellular trap literature suggests that macrophage extracellular traps are similar to NETs in that they are the product of a programmed macrophage cell death process, contain many of the same components as NETs, and play both antimicrobial and detrimental roles in different settings of inflammation and infection (Doster, Rogers et al. 2017). Finally, extracellular traps can be produced by monocytes after stimulation with several of the same NETosis-associated stimuli. Monocyte-derived extracellular traps share similar components such as neutrophil elastase, myeloperoxidase, and citrullinated histones (Granger, Faille et al. 2017). Monocyte-derived extracellular traps can also contribute to coagulation, and are thought to be implicated in thrombosis, infection, and inflammation (Granger, Faille et al. 2017).

**Clearance of NETs**

NETs are thought to be cleared from the circulation via degradation by DNase I (Hakkim, Furnrohr et al. 2010) and macrophage phagocytosis (Farrera and Fadeel 2013). NETs were determined to be stable for up to 90 hours when isolated from serum; however, in serum, NETs are degraded in a time- and concentration-dependent manner. Further investigation into this observation led to the conclusion that this degradation is done by DNase I, an extracellular endonuclease mainly produced in the pancreas (Hakkim, Furnrohr et al. 2010). However, incubation of NETs with DNase I at the physiological concentration of 20 ng/ml and serum from healthy donors at 10% or 20% only triggered partial degradation of NETs, indicating other factors contribute to physiological NET clearance (Farrera and Fadeel 2013). Macrophage phagocytosis of pathogens is a well described mechanism of pathogen clearance; macrophages also clear apoptotic neutrophils with ultimately an anti-inflammatory effect (Greenlee-Wacker 2016). Examination of the potential of macrophages to clear NETs from the circulation yielded similar
results: macrophages can clear NETs from the circulation, and this process is facilitated by processing of NETs by DNase I (Farrera and Fadeel 2013). Binding to C1q, which is the recognition factor that activates the classical complement pathway, results in either phagocytosis or complement activation; interestingly, C1q binding to NETs promoted the engulfment and macrophage clearance of NETs (Farrera and Fadeel 2013).

Notably, none of the physiologically relevant NET-clearance mechanisms described above initiated an immune response, in contrast to experimental NET clearance conditions which resulted in the release of type I interferons (Farrera and Fadeel 2013). Recently, phenotype differences in the macrophage response to NETs have been described. Specifically, following NET degradation, M1 macrophages demonstrated cell death and subsequent peptidyl arginine deiminase 4-mediated nuclear decondensation and release. Caspases then activated the same M1 macrophages to clear the extracellular DNA that had been released in a DNase-mediated manner, and the DNA was cleared within 24 hours. In contrast, M2 macrophages induced a pro-inflammatory response when presented with NET degradation. The authors of this study discuss that the fluctuation within the M1 response, as well as difference in phenotype responses, is characteristic of the “double-edged sword” property of NETs seen throughout the literature (Nakazawa, Shida et al. 2016).

Detecting NETs

Detection of NETs poses a challenge, as it is difficult but crucial to differentiate NETs, which are largely comprised of DNA, from cell-free DNA. To quantify NETs in vivo, a sandwich enzyme-linked immunosorbent assay (ELISA) technique has been described (Kessenbrock, Krumbholz et al. 2009, Sayah, Mallavia et al. 2015). An ELISA detects proteins by coating a plate with specific antibodies for the protein of interest; after washing the samples, a detection antibody is added and the intensity of the resulting colorimetric change is used to calculate the amount of protein of interest in the sample. In the case of NETs, a capture antibody for a specific neutrophil granule component, such as myeloperoxidase or neutrophil elastase, can be used in conjunction with a detector antibody specific for DNA; thus, the resulting signal is specific for granule component-DNA complexes.

Other methods of in vivo NET detection include immunofluorescence for DNA and NET components, such as myeloperoxidase, neutrophil elastase, citrullinated histone 3, cathepsin G,
and LL-37. Co-localization of these proteins with extracellular DNA has been used to determine the presence of NETs. As there has been a surge of interest in NETosis, novel methods of NET quantification have emerged in the literature. A technique for flow cytometric analysis of NETs in human blood samples was recently published, which employs antibodies against well-described NET-associated proteins, such as neutrophil granular components and citrullinated histones (Gavillet, Martinod et al. 2015).

*In vitro*, NETosis is generally determined through stimulation of isolated neutrophils with NETosis agonists and measurement of subsequent DNA release using a cell impermeable nucleic acid dye, such as SYTOX Green (Hakkim, Fuchs et al. 2011, Parker, Dragunow et al. 2012, Douda, Khan et al. 2015). The necessity to differentiate between extracellular DNA and NETs makes the development of high-throughput and automatic methods for quantification of NETs challenging. However, recent advances using imaging and 3D confocal microscopy have shown that high-throughput quantification of NETs is an achievable goal (Brinkmann, Goosmann et al. 2012, Kraaij, Tengstrom et al. 2016).

**NETs as a part of the innate immune response**

NETosis has been described in the literature as a “double-edged sword” (Kaplan and Radic 2012), meaning that NETs have both beneficial and detrimental effects. Neutrophils are the first responders of the immune system; thus, NETs contribute to the first line of defense against invading pathogens. NETs physically trap pathogens in the web-like structure of extracellular chromatin; this structure is necessary for the antimicrobial proteins to kill pathogens, as the structure ensures high concentrations of antimicrobial components and cytotoxic histones in contact with the pathogen (Brinkmann, Reichard et al. 2004).

A variety of gram-positive and gram-negative bacteria can induce NETosis; as per a systematic review by Hoppenbrouwers et al., the most potent bacterial inducers were *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium bovis* (Hoppenbrouwers, Autar et al. 2017). NETosis in response to gram-positive bacteria has been visualized and shown to be rapid, during the neutrophil crawling phase, and via vital NETosis thereby resulting in both NETs and anuclear yet functional neutrophils (Yipp, Petri et al. 2012). This discovery is especially important because it demonstrates that NETosis occurs within minutes of pathogenic invasion, and
creates defense mechanisms in the form of NETs while retaining the original defense capabilities of neutrophils. NETosis also occurs in response to the *Candida albicans* yeast and hyphal cells, demonstrating an antifungal role for NETs (Urban, Reichard et al. 2006, Byrd, O'Brien et al. 2013). NETs have recently been shown to contribute to the antiviral immune response. NETosis can occur in direct response to viral infection via TLR recognition (Saitoh, Komano et al. 2012), or indirectly through the production of pro-inflammatory cytokines by infected cells (Mogensen and Paludan 2001) and the production of interferons which act to prime mature neutrophils to undergo NETosis (Martinelli, Urosevic et al. 2004).

Not all neutrophils undergo NETosis; only an approximate 20-25% of neutrophils presented with NETosis-inducing stimuli actually form NETs (Yipp and Kubes 2013). This may be because neutrophils can sense pathogen size and undergo NETosis in response to large pathogens *in vivo* (Branzk, Lubojemska et al. 2014). Another possible reason for this is difference in function and characteristics of neutrophil subpopulations. Shaul and colleagues describe two distinct neutrophil subtypes, the N1 tumor-inhibitory phenotype and the N2 tumor-promoting phenotype (Shaul, Levy et al. 2016). Neutrophils are thought to retain plasticity with regard to the specific neutrophil phenotype they express, and the determination of phenotype expression is thought to be due to components of the surrounding microenvironment. Specifically, transforming growth factor-β has been shown to play an important role in neutrophil phenotypes in tumors: inhibition of transforming growth factor-β results in increased tumor-associated neutrophils of the anti-tumor (N1 phenotype) and reduction of tumor growth, and lack of inhibition of transforming growth factor-β results in N2 pro-tumor neutrophils and associated tumor growth (Fridlender, Sun et al. 2009). N2 neutrophils can induce T-cell tolerance thereby contributing to immunosuppression and subsequent tumor growth, and N1 neutrophils contribute to inhibition of tumor growth by high levels of tumor necrosis factor-α expression and high intercellular adhesion molecule-1 (Shaul, Levy et al. 2016). Of note for this thesis, antitumor neutrophils are associated with high levels of NETs and associated killing of tumor cells; this is thought to be due to the presence of type I interferons, as neutrophils deficient in type I interferon demonstrated suppressed levels of NETs and reduced ability to kill cancer cells (Andzinski, Kasnitz et al. 2016). Altogether, this data suggests that the N1 phenotype of neutrophils may undergo NETosis more readily than the N2 phenotype, and this could explain discrepancies in neutrophils that undergo NETosis and those that do not.
NETs in inflammation, lung disease, and lung injury

Although beneficial in the context of pathogen trapping, NETS in excess or in the wrong context can be detrimental. The cytotoxicity of the exposed histones, which is beneficial for the neutralization of trapped pathogens, can lead directly to epithelial and endothelial cell death (Saffarzadeh, Juenemann et al. 2012). In ALI, extracellular histones result from complement activation and contribute to severity of lung injury; NETs are thought to be both products of complement activation as well as mediators of extracellular histone-induced lung injury (Bosmann, Grailer et al. 2013).

NETs can contribute to innate immune activation by interacting with complement factors. The complement system is a part of the innate immune system which acts to promote phagocytosis, contribute to inflammation through neutrophil and macrophage recruitment, and attacking bacterial cell walls through the membrane attack complex. There are three complement pathways: the classical complement pathway, the alternative complement pathway, and the lectin pathway. NETs have been shown to activate the alternative complement pathway in an in vitro model of anti-neutrophil cytoplasmic antibody-associated vasculitis, although this study notably demonstrated that the ability of NETs to induce complement activation was not specific to vasculitis-induced NETs, but could also occur due to PMA- or lipopolysaccharide-induced NETs (Wang, Wang et al. 2015). Complement opsonisation, and specifically the actions of complement receptor 1, activates NETosis in a variety of bacterial challenges (Palmer, Damgaard et al. 2016). Complement activation is a well-described mediator of graft injury, contributing to ischemia-reperfusion injury and long-term chronic allograft dysfunction (Sheen and Heeger 2015).

NETs can also contribute to vascular occlusion and thrombosis by providing a scaffold for platelets and thrombosis-associated proteins, as well as by forming clots in the vasculature (Fuchs, Brill et al. 2010, Jimenez-Alcazar, Rangaswamy et al. 2017). Heparin, a known anti-coagulant, disrupts the structures of NETs by releasing histones from the extracellular chromatin (Fuchs, Brill et al. 2010). NETs may form in response to pathogen invasion, with the consequence of contributing to thrombosis of the vasculature; this is thought to be a possible link between thrombosis and infection (Fuchs, Brill et al. 2010) which illustrates the fine balance between beneficial and detrimental NETs. Platelet-mediated NETosis is thought to contribute to thrombi
formation, as platelets are activated in the context of thrombosis and are important triggers and mediators of NETosis (Carestia, Kaufman et al. 2016).

Autophagy-mediated NETosis has been shown in vitro to promote lung fibroblast function and activate differentiation into myofibroblasts. Neutrophils stimulated with typical NETosis agonists as well as a variety of agents involved in fibrosis, including cigarette smoke, underwent NETosis and induced lung fibroblast activation (Chrysanthopoulou, Mitroulis et al. 2014). NETosis in this context was mediated by autophagy, and NETs augmented the fibrotic effects of myofibroblasts.

The propagation of neutrophil recruitment, activation, and subsequent inflammation is another way by which NETs are implicated in injury. Alveolar and bronchial epithelial cells demonstrated significantly increased secretion of IL-8 and IL-6 when stimulated by NETs, specifically in the apical compartment of polarized cells, indicating that NETs could propagate a pro-inflammatory environment in the lungs and contribute to a chemotactic gradient encouraging neutrophil transepithelial migration (Sabbione, Keitelman et al. 2017).

NETs are associated with many instances of lung disease. NETs have been shown to be associated with higher severity of COPD, and many neutrophils undergo NETosis in the airways of COPD patients during acute exacerbation; approximately 90% of patients with exacerbated COPD demonstrated high volume of NETs in sputs (Obermayer, Stoiber et al. 2014, Grabcanovic-Musija, Obermayer et al. 2015). NETs are also found in high volumes in the sputs of stable COPD patients, and higher NET quantity in COPD patients is associated with greater airflow limitation (Grabcanovic-Musija, Obermayer et al. 2015). NETs are potential agents in interstitial lung disease (ILD), as patients with ILD-associated pathologies were found to have increased levels of circulating LL37 and cell-free DNA, as well as higher rates of neutrophils undergoing NETosis; decrease of DNAse I activity and subsequent maintained levels of NETs are though to contribute to the propagation of ILD in patients with autoimmune disease (Zhang, Shu et al. 2014).

Cystic fibrosis (CF) lung disease is caused by a mutation in the CF transmembrane conductance regulator gene, and is characterized by chronic inflammation, increased neutrophilia of the airways, and bacterial colonization. NETs are a major component in the sputum from CF patients (Manzenreiter, Kienberger et al. 2012, Dwyer, Shan et al. 2014). Worse pulmonary function in CF patients was associated with higher levels of NET-derived extracellular DNA (Marcos, Zhou-Suckow et al. 2015). It is possible that NETs contribute to the pathophysiology of CF by initially acting as beneficial antifungal or antibacterial agents, but as the disease progresses,
NETs accumulate in the airways causing inflammation and airway obstruction (Marcos, Zhou-Suckow et al. 2015).

NETs have been implicated in many types of ALI. For instance, NETs are detectable in different models of ventilator-induced lung injury, although the mechanisms by which they are formed in this context remain unclear (Rossaint, Herter et al. 2014, Yildiz, Palaniyar et al. 2015). In transfusion-related acute lung injury, a complication of blood transfusion, NETs are produced in a platelet-dependent manner and serve as therapeutic targets (Caudrillier, Kessenbrock et al. 2012, Thomas, Carbo et al. 2012). Trauma-associated lung injury is mediated by circulating histones, which can induce NETosis in this context and lead to lung neutrophilia and thrombosis (Abrams, Zhang et al. 2013). NETs in circulation are strongly associated with bacteria in tracheal aspirate, a marker for preclinical infection due to aspiration (Hirose, Hamaguchi et al. 2014). NETs are also implicated in ALI associated with influenza pneumonitis, and NETosis was strongly induced in neutrophils isolated from mice challenged with influenza A virus H1N1 strain PR8 (Narasaraju, Yang et al. 2011).

Of note is the finding that NETs are pathogenic in PGD (Sayah, Mallavia et al. 2015). This study examined both a hilar clamp and orthotopic transplantation model in mice; the hilar clamp model induced ischemia-reperfusion injury without transplantation, and the orthotopic lung transplant model represented the full lung transplant process. NETs were pathogenic in both settings, indicating that NETosis is triggered by the ischemia and reperfusion processes (demonstrated in the hilar clamp model), as well as recipient response to allograft implantation (demonstrated in the orthotopic transplantation model). Interestingly, this study found that NETs were increased in the circulation after hilar clamping but not in the BAL fluid; meanwhile, NETs were significantly increased in the BAL fluid from a prolonged cold ischemic model of transplantation when compared to a control transplantation model, and neither transplantation model demonstrated high levels of NETs in the plasma.

NETs in autoimmunity

NETs have been described in the context of transplant-related alloimmunity. However, it is important to note that NETs can be generated in response to self-antigens, and not solely as a result of external factors. NETs are thought to play a role in a variety of autoimmune as well as
autoinflammatory diseases. In the case of autoinflammatory disease, NETosis generally promotes an inflammatory state by propagating a cycle of neutrophil recruitment and activation. For instance, in ulcerative colitis patients, NETs were found in excess in colon mucosa (Bennike, Carlsen et al. 2015). Interestingly, NETs are produced in gout and associated with the release of inflammatory cytokine IL-1β (Mitroulis, Kambas et al. 2011); however, other reports indicate that NETs seem to regulate inflammation in gout by degrading IL-1β (Schauer, Janko et al. 2014). NETs have also been implicated as playing a role in the pathogenesis of small vessel vasculitis (Kessenbrock, Krumbholz et al. 2009).

Rheumatoid arthritis is characterized by the development of autoantibodies to citrullinated proteins and to rheumatoid factor, and increased neutrophils and inflammatory cytokines in the synovial fluid; NETosis was found to be elevated in neutrophils from the synovial fluid in rheumatoid arthritis patients as well as peripheral blood, and NETosis can be mediated by rheumatoid arthritis autoantibodies and rheumatoid arthritis autoantibodies can be developed against NET components in this patient population (Khandpur, Carmona-Rivera et al. 2013).

Sex differences in immunity, inflammation, and NETs

Sex differences in systemic inflammation as well as inflammation specific to lung transplantation have been described in the literature (Breithaupt-Faloppa, Ferreira et al. 2016, Klein and Flanagan 2016). There are well defined differences between male and female innate and adaptive immune systems; in general, females present a more robust response to immune challenge. This is thought to be related to the presence of sex-related hormones. For instance, lipopolysaccharide-challenged reproductive-aged female rats demonstrate a greater ability for phagocytosis by circulating neutrophils than males, pre-, and post-reproductive aged females (Spitzer 1999). Another difference in the innate immune system is the expression of pattern recognition receptors: TLR7, which is a toll-like receptor that plays a role in autoimmunity and antiviral defense, is expressed more highly in females than males, and in vitro female peripheral blood mononuclear cells and plasmacytoid dendritic cell exposure to TLR7 results in greater production of interferon-α than male peripheral blood mononuclear cells and plasmacytoid dendritic cells (Klein and Flanagan 2016).
A rat model of DCD and ischemia-reperfusion injury demonstrated attenuated vascular resistance and retained oxygen transport ability in females, compared to males (Mrazkova, Lischke et al. 2016). Breithaupt-Faloppa et al. demonstrated in a rat model of brain death that female rats and ovariectomized female rats retained leukocyte counts for up to 6 hours after brain death, while male rats demonstrated leukopenia 6 hours after brain death (Simao, Ferreira et al. 2016); the authors hypothesized that the female retention of leukocytes was due to the ability of estradiol and progesterone to attenuate neutrophil apoptosis (Molloy, O'Neill et al. 2003). Estradiol also demonstrated an immunomodulatory protective effect in the lungs against intestinal ischemia-reperfusion induced acute lung inflammation (Breithaupt-Faloppa, Thais Fantozzi et al. 2014). This evidence points overall towards a generally protective effect of female sex hormones in lung inflammation and injury; however, the role of female hormones in lung transplantation is not definitive, as adverse recipient outcomes from lung transplants with female donors have been reported (Chaney, Suzuki et al. 2014, Taghavi, Jayarajan et al. 2014).

Sex differences have been described in lung disease as well as lung transplant-related injury. In a cohort of 32,766 CF patients who did not receive a transplant, survival was significantly worse for women than men, with a median survival of 36.0 years in females and 38.7 years in males (p<0.001), and being a female was a significant risk factor for death (Harness-Brumley, Elliott et al. 2014). With regard to respiratory infections, females developed infection to CF-related pathogens such as *P. aeruginosa* and *Aspergillus* earlier in life and with worse survival. The differences in CF survival between genders is thought to be largely due to estrogen, as estrogen can be pro-inflammatory (i.e. by upregulating IL-17, which correlates with the severity of lung inflammation in CF) as well as act to suppress lactoferrin, which can increase bacterial burden in experimental CF models (Sweezey and Ratjen 2014). Other fibrotic diseases, such as pulmonary fibrosis, are more prevalent in males than in females; however, a review of animal studies in the literature demonstrates conflicting results of whether males or females suffer from more severe pulmonary fibrosis-related lung injury (Townsend, Miller et al. 2012). COPD has also been described as being more severe in female patient populations than male, and pulmonary hypertension is two to four times more prevalent and develops earlier in females than in males (Townsend, Miller et al. 2012).

As with many aspects of NETs, the interplay between hormones and NETosis is under investigation. However, gender differences in NETosis have been documented. For instance,
neutrophils isolated from pregnant women (throughout which the immune system is in a pro-inflammatory state) were more likely to undergo NETosis than neutrophils from non-pregnant women; furthermore, the susceptibility to undergo NETosis in vitro increased as the gestation progressed (Giaglis, Stoikou et al. 2016). Granulocyte colony-stimulating factor was shown to play an important role in promoting a pro-NETosis state in the neutrophils of pregnant women, and neutrophils isolated from non-pregnant controls could be primed to undergo greater NETosis in vitro with the addition of granulocyte colony-stimulating factor (Giaglis, Stoikou et al. 2016). Circulating NETs have also been documented to differ between genders in patients with the autoimmune disease relapsing remitting-multiple sclerosis. Higher levels of NETs were detected in male relapsing remitting-multiple sclerosis patients, although NETs may not contribute to the pathogenesis of relapsing remitting-multiple sclerosis and rather serve as an indicator of underlying differences in the immune and autoimmune response between genders (Tillack, Naegele et al. 2013).

Rationale for formulating the hypothesis

Neutrophils are well-described mediators of acute lung injury related to transplantation. Recently, NETosis has been implicated as a process by which neutrophils can affect injury in the lungs. Despite the many recent advances in the fields of lung transplantation and NETosis research, the role of NETosis throughout the lung transplantation process is not fully defined. NETs in donor lungs have not yet been well described, nor have the relationship of NETs and lung transplant recipient outcomes beyond the development of PGD. However, the literature suggests that NETs play an important role in this context of affecting and predicting lung injury.

Lung injury in the context of lung transplantation is multifactorial and broad: injury can be sustained by the donor lung prior to any surgical procedure, during the lung preservation period, as a result of the reperfusion process, due to graft rejection, or from comorbidities such as pneumonia. As such, in order to mitigate the possibility of injury throughout this process, it is imperative to understand the biological processes that are occurring. While a complete understanding of such a complex injury process will continue to evolve, finding key effectors or indicators of lung injury can assist in developing treatments or prevention strategies.
In addition to their known antimicrobial function, NETs have been implicated throughout the literature as contributors to a variety of inflammatory and injurious pathologies. Several inflammatory mediators described as inciting factors of NETosis – for example, CXCL8 – are also strongly implicated in the development of lung transplant-related injury. Therefore, this thesis tests the following overall hypothesis under 4 aims, that are presented in each of the 4 following chapters.

Hypothesis

**Hypothesis:** NETs are important markers, as well as effectors, of lung transplant-related injury.

**Aim 1:** to determine if the clinically relevant large animal (swine) model of lung transplantation and related lung injury was a suitable platform to study NETs (Chapter 2: Section 2.2-4).

**Aim 2:** to test whether or not NETs form in clinically relevant lung injury models – specifically, donor lung injury, ischemia reperfusion injury, and severe aspiration injury – and assess their association with the degree or type of lung injury (Chapter 2: Section 2.2-4).

**Aim 3:** to determine if EVLP is a suitable platform to study NETosis, by testing whether NETs can be detected in perfusate and whether detectable NETs could provide valuable information about the lungs (Chapter 2: Section 2.4; Chapter 3: Section 3.2).

**Aim 4:** to see if agents which have been described as anti-inflammatory or beneficial in the context of lung transplantation act by exerting a suppressive effect on NETs or NETosis (Chapter 2: Section 2.2-3)

**Aim 5:** to confirm that isolated swine neutrophils undergo NETosis comparably to human neutrophils (Chapter 2.5)

**Aim 6:** to translate the findings of the large animal studies to human studies to assess the clinical relevance (Chapter 3).
Chapter 2: Animal Studies
2.1 Rationale, hypotheses and research aims

The existing evidence implicates NETs in acute lung injury, and demonstrates their pathogenicity in PGD. The detrimental role played by NETs in lung injury, as well as the knowledge that many of the active inflammatory pathways in lung injury and ischemia-reperfusion injury are also upstream of NETosis, inspired the design of this series of large animal studies.

These studies aimed to characterize the presence and significance of NETs in vivo in various animal models of transplant related-injury, and explore possible ways to mitigate NET-related injury. The specific aims addressed in this series of large animal studies, as described in Chapter 1: Hypothesis, all explore the hypothesis that NETs are important effectors and markers of lung transplant-related injury.

The first aim addressed in this chapter was to confirm that isolated swine neutrophils undergo NETosis comparably to human neutrophils (Chapter 2.2). This study aimed to address whether the results from what was observed in the large animal models could be extrapolated to humans on a cellular level.

The next aim was to determine if the swine model of lung transplantation and related injury is a suitable platform to study NETs in lung transplantation. This aim is addressed in this chapter throughout three different, clinically relevant studies of large animal models (Chapter 2: Section 2.3-5). The first study was a swine model of ischemia reperfusion injury, wherein an orthotopic lung transplantation was followed by 72 hours of survival to examine early graft function post-transplant. The second study was a model of clinically relevant aspiration injury, which induced severe enough injury that animals were supported using ECMO. Aspiration injury is well-documented in lung transplantation, and can occur in organ donors, compromising the quality of the donor lungs, as well as in recipients after transplantation. Furthermore, gastric aspiration induces neutrophil-mediated acute lung injury (Kennedy, Johnson et al. 1989). Thus, an aspiration injury model is highly relevant to the study of NETosis in lung transplantation. The use of ECMO increases the clinical relevance of this model, as ECMO is increasingly used as a bridge to transplantation as well as for perioperative support and management.

The third study compared different models of donor lung injury, including severe donor lung injury, and compared their performance during EVLP. In all of these studies, biological samples including EVLP perfusate, BAL fluid, plasma, and lung tissue were collected and
analyzed for NETs at various time point throughout the experiments. NETs were posited to be readily detectable in these samples, indicating that they could be studied using large animal models.

The third aim was to test whether NETs form in clinically relevant lung injury models (donor lung injury, ischemia reperfusion injury, severe aspiration injury), and their association with the degree and type of injury (Chapter 2: Section 2.3-5). This aim was achieved through analysis of NETs detected in the aforementioned swine models of lung injury, with regard to the degree and type of experimental lung injury. NETs were postulated to be detected in higher quantities in more severely injured lungs.

The fourth aim was to determine if EVLP is a suitable platform to study NETosis, by testing whether NETs can be detected in perfusate and whether detectable NETs could provide valuable information about the lungs (Chapter 2: Section 2.5). This aim was explored through the study which compared three different models of donor lung injury and subsequent EVLP. The first model was a cardiac death model followed by prolonged warm ischemic time prior to lung retrieval. This model does not mimic the clinical setting, as the warm ischemic time is minimized clinically; however, this model was important to include experimentally as it served as the most severe injury and was a clinically relevant mode of donor death. In the second model, the injury was induced by prolonged cold ischemic storage. The third group served as the control group, including minimal cold ischemic time. NETs were posited to be detectable in EVLP perfusate, thereby indicating their formation in the donor lung prior to transplantation. Furthermore, quantity of detectable NETs in the perfusate were posited to be associated with lung injury severity, with higher levels of NETs being associated with worse lung injury and lung function.

The fourth aim was to see if agents which have been described as anti-inflammatory or beneficial in the context of lung transplantation act by exerting a suppressive effect on NETs or NETosis (Chapter 2: Section 2.3-5). The ischemia-reperfusion injury and aspiration injury studies, described above and in Chapter 2: Section 2.3-4, were designed to test questions regarding the effects of treatments: alpha-1 antitrypsin for ischemia-reperfusion injury, and surfactant for aspiration injury. Importantly, while these studies provided a platform to assess the effect of potentially beneficial therapeutics on NETs in lung transplantation-related injury models, NETosis was a secondary aim within these studies and, as such, the study designs lack a healthy control arm.
Alpha-1 antitrypsin is an acute phase protein, which has been shown to have broad anti-inflammatory and anti-apoptotic effects. One major mechanism of alpha-1 antitrypsin is to act in a suicidal manner by binding and inhibiting neutrophil elastase. Neutrophil elastase is a known effector as well as regulator of NETosis (Papayannopoulos, Metzler et al. 2010); thus, its inhibition by alpha-1 antitrypsin could contribute to suppression of NETosis as well as mitigate the effects of NETs that do form. Therefore, this study was designed to assess if alpha-1 antitrypsin in the three-day survival PGD model suppresses NETosis and related lung injury outcomes.

The second potentially beneficial agent that was explored in order to address Aim 4 was surfactant therapy in the aspiration injury model. Surfactant treatment has been shown in an aspiration injury model to improve lung function ex vivo (Nakajima, Liu et al. 2017). In this study, surfactant was posited to improve lung function as well as mitigate the inflammatory effects of NETs.
2.2 In vitro studies

**Aim 5:** to confirm that isolated swine neutrophils undergo NETosis comparably to human neutrophils

To assess the effects of alpha-1 antitrypsin on a cellular level, a study was designed to induce NETosis in neutrophils isolated from pigs in the 72-hour lung transplant survival study described below (Chapter 2.3). Before beginning, a pilot study was done to ensure pig neutrophils responded to NETosis stimuli in the same manner as human neutrophils. Due to the lack of typical response of healthy control pig neutrophils to NETosis agonists (described below, in Results), the sample size for the study assessing NETosis from transplanted pig neutrophils was ultimately small.

**Methods**

Blood samples were taken from the transplant recipient animals 24 and 48 hours after reperfusion, and neutrophils were isolated using Polymorphprep (Axis-Shield), as described in the literature (Douda, Khan et al. 2015). Blood samples from healthy control pigs were also taken and neutrophils isolated and stimulated in the same manner for comparison.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
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<tr>
<td>Control</td>
<td>3</td>
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<tr>
<td>Treatment Transplant – 24 h</td>
<td>1</td>
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<tr>
<td>Treatment Transplant – 48 h</td>
<td>2</td>
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<tr>
<td>Control Transplant – 24 h</td>
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<tr>
<td>Control Transplant – 48 h</td>
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*Table 1. NETosis assays study groups*

Briefly, blood samples were collected into tubes coated with ethylenediaminetetraacetic acid to prevent coagulation. After collection and transportation to the lab, blood was coated onto an equal volume of Polymorphprep and spun down at 400 g for 35 minutes. The layer containing polymorphonuclear cells was collected and lysed using a hypotonic solution (0.2% NaCl),
followed by addition of equal volume of NaCl with Hepes buffer (20 mM) to stop the lysis. The cells were then washed twice (0.85% NaCl with 10mM Hepes). Finally, the cells were suspended in Rosewell Park Memorial Institute medium and counted. Sytox Green dye was added to the cells in media, and 500,000 cells were plated in each well on a 96-well plate. The NOX-dependent agonist PMA as well as NOX-independent agonists ionomycin and calcium ionophore A23187 were added to the cells as well as 10% Triton X as a positive control. Baseline fluorescence from the Sytox Green, representing extracellular DNA, was captured using a fluorescence reader, and cells were incubated at 37°C with subsequent fluorescence readings every 30 minutes thereafter. The results are reported as % DNA release, as determined by the fluorescence reading of Sytox Green for each well relative to the Triton X positive control. Neutrophils were stimulated with PMA, ionomycin, and calcium ionophore A32817 in duplicate at varying concentrations. A control well was included in each study to gage background from normal neutrophil cell death over the course of each experiment. The results are reported as the mean % DNA release for each stimulus or control.

Surprisingly, there was little response of pig neutrophils to typical NETosis stimuli: the calcium ionophores produced a relatively weak response to stimuli, while PMA resulted in almost no change from the negative control (described below, see Results). Titration experiments in agonist concentration (n=2) produced little change in the response of pig neutrophils (see Results). To address if this was due to a NET-inhibiting factor from the transplant process or whether it was due to a species difference, neutrophils from healthy pig donors (n=2) and healthy human donors (n=2) were isolated and stimulated for NETosis simultaneously. PMA was assessed in concentrations ranging from 50 nM – 600 nM, ionomycin was added in concentrations of 4 μM – 24 μM, and the ionophore A23187 was assessed from 2 μM – 12 μM; all agonists were added in concentrations greater than typically necessary to stimulate NETosis in healthy human controls (Douda, Khan et al. 2015). The neutrophil isolation from pig blood samples was achieved as described above, and as described in the literature (Douda, Khan et al. 2015) for the human blood samples.
Results

Neutrophils isolated from healthy control pigs as well as transplanted pigs who received either alpha-1 antitrypsin or saline placebo before reperfusion and during the 72 hours after reperfusion demonstrated dampened responses to typical NETosis agonists. Specifically, neither PMA, ionomycin, or calcium ionophore A23187 induced a response similar to that seen in the literature, in pigs who received transplants as well as three healthy control pigs.

a) Swine neutrophils stimulated with PMA

b) Swine neutrophils stimulated with A23187

c) Swine neutrophils stimulated with ionomycin
NETosis assays in neutrophils isolated from swine lung transplant recipients and healthy controls.

Neutrophils were isolated from swine lung transplant recipients as well as healthy swine controls. Isolated neutrophils were stimulated using agonists of NOX-independent NETosis (calcium ionophore A23187, ionomycin) and NOX-dependent NETosis (PMA). NETosis was determined by Sytox green measurements, representative of extracellular DNA, compared to the positive control (10% Triton). Neither PMA (a), ionophore A23187 (b), nor ionomycin (c) induced the expected robust response to agonists.

Figure 1. Transplanted pig NETosis assays

Neutrophils isolated from healthy control pigs were stimulated with a variety of concentrations of PMA and calcium ionophore A23187 on two separate occasions, shown below in Figure 12. Neutrophils stimulated with PMA demonstrated negligible activation at any concentration. Neutrophils stimulated with A23187 showed some response at high levels of the agonist; however, this response differed from what has been documented in human neutrophils with regard to strength of response as well as kinetics.

**NETosis assays from healthy control swine neutrophils demonstrate sluggish response to typical NETosis agonists.** Swine neutrophils isolated from healthy control pigs were stimulated with PMA at varying concentrations (a) and calcium ionophore A23187 at varying concentrations (b). Both agonists resulted in sluggish neutrophil response.
When neutrophils isolated from healthy pigs and healthy human donors were stimulated for NETosis on the same day, using the same concentrations of the same reagents, a difference was seen in the response of the human neutrophils compared to the swine neutrophils. Notably, the release of Sytox Green dye–used as a marker of NET release–from the human neutrophils followed very typical time courses, described in the NETosis literature (Douda, Khan et al. 2015), for all agonists at all concentrations. However, the response of the pig neutrophils to PMA was almost non-existent, and to the ionophores was sluggish in comparison to human neutrophils.

a) Neutrophils stimulated with varying concentrations of PMA

![Graph showing DNA release in pig neutrophils](image1)

![Graph showing DNA release in human neutrophils](image2)

b) Neutrophils stimulated with varying concentrations of ionomycin

![Graph showing DNA release in pig neutrophils](image3)

![Graph showing DNA release in human neutrophils](image4)

c) Neutrophils stimulated with varying concentrations of A23187

![Graph showing DNA release in pig neutrophils](image5)

![Graph showing DNA release in human neutrophils](image6)
NETosis assays from healthy swine and human controls demonstrate difference in neutrophil response to typical NETosis agonists. Neutrophils isolated from healthy swine controls and healthy human subjects were stimulated with NOX-dependent NETosis agonist PMA and NOX-independent NETosis agonists ionomycin and calcium ionophore A23187. Swine and human neutrophils demonstrated different responses to PMA (a), ionomycin (b), and calcium ionophore A23187 (c).

Figure 3. NETosis assays with pig and human neutrophils

Discussion

This study aimed to confirm that isolated swine neutrophils undergo NETosis in a manner that is comparable to isolated human neutrophils, in order to translate what was observed in large animal models of lung injury to humans on a cellular level. These studies show that pig neutrophils, from both transplant recipient pigs as well as healthy control pigs, demonstrate a suppressed response to NETosis agonists compared to the typical human neutrophil response. A response was not elicited when agonists were titrated, and a difference in response between swine and human neutrophils was further supported when human and control pig neutrophils were isolated and stimulated for NETosis in the same experiment.

These results strongly suggest a species difference in the neutrophil response to NETosis agonists. Other groups have described a difference in the response of porcine neutrophils to fMLP compared to human neutrophils, although porcine neutrophils responded similarly to human neutrophils when presented with PMA and calcium ionophore A23187 (Fletcher, Stahl et al. 1990). Porcine neutrophils have been reported as smaller but with similar morphology to human neutrophils; pig neutrophils responded similarly to calcium ionophore A23187, and human alpha-1 protease inhibitor inhibited porcine neutrophil elastase as it does human neutrophil elastase (Brea, Meurens et al. 2012).

Our observations that purified porcine neutrophils do not undergo NETosis in a manner similar to purified human neutrophils is not in agreement with the reviewed literature; however,
the body of literature surrounding this topic is fairly small. It is possible that pig neutrophils from the Yorkshire strain used in these experiments require priming prior to NETosis, as one study suggests that both porcine and human neutrophils demonstrate an enhanced response activation after priming with platelet activating factor (Yaffe, Xu et al. 1999). Different rat strains have been reported to experience differences in neutrophil infiltration in lung inflammation (Zhang, Lin et al. 2011); it is possible that the strain of pigs used in our studies is different than those used by other groups, and thus our results are indicative of a strain difference in neutrophil response.

NE-DNA complexes representative of NETs were detected in a variety of pig lung transplantation and EVLP models, suggesting that swine neutrophils produce NETs under injurious conditions. However, NETosis was not observed in isolated neutrophils from transplanted pigs stimulated with high concentrations of different, well-described NETosis stimuli. Notably, NETosis was observed to be suppressed in neutrophils isolated from five pigs included in the alpha-1 antitrypsin study, but NETs were detectable in the BAL from the same animals at 72 hours after transplant. This suggests that swine neutrophils undergo NETosis differently than human neutrophils, either via a different mechanism (Fletcher, Stahl et al. 1990) or only after a much greater immune challenge than was presented in this study. However, NETs have been described in the BAL samples of clinical transplant patients (Sayah, Mallavia et al. 2015), implying that the severity of injury sustained during lung transplantation is enough to induce NETosis in humans. The animal models under discussion closely mimic clinical lung transplantation-related injury, and NETs were detected after injury in these models, suggesting that it is not the severity of the injury that underlies the observed difference in human and pig NETosis.

Neutrophils have been shown to possess the ability to migrate to and from the lung (Wang, Hossain et al. 2017). Another possible explanation for the presence of NETs in porcine lungs after injury, but observed reduced ability of porcine neutrophils to undergo NETosis, is that neutrophils capable of NETosis migrated to the lung in the lung transplant-related injury models. Therefore, NETs were detected in the lungs after lung injury, but NETosis was not observed in neutrophils isolated from systemic blood samples. This is speculation; however, this idea is supported by the observation that patients who developed PGD demonstrated higher levels of NETs in post-transplant BAL samples than patients who did not, but pre-transplant and post-transplant levels of NETs in the plasma did not differ in either group (Sayah, Mallavia et al. 2015).
It is important to remember that porcine neutrophils *in vivo* in our studies seemed to respond to lung injury by producing NETs, which were measured in a variety of injury types as NE-DNA complexes. Thus, it seems that the porcine neutrophils differ from human neutrophils only once isolated *in vitro*. This could be because the magnitude and type of injury required for porcine neutrophils to undergo NETosis is not replicated by the agonists used *in vitro*. Similarly, there could be priming factors present *in vivo* which are required by porcine neutrophils for NETosis, which were not present in these *in vitro* studies. This difference in porcine response to NETosis agonists is important, as it demonstrates one of the challenges of translational research.
2.3 NETs in ischemia reperfusion injury

**Aim 1:** to determine if the clinically relevant large animal (swine) model of lung transplantation and related lung injury was a suitable platform to study NETs

**Aim 2:** to test whether or not NETs form in clinically relevant lung injury models – specifically, donor lung injury, ischemia reperfusion injury, and severe aspiration injury – and assess their association with the degree or type of lung injury

**Aim 4:** to see if agents which have been described as anti-inflammatory or beneficial in the context of lung transplantation act by exerting a suppressive effect on NETs or NETosis

**Methods**

A study was performed assessing the effects of alpha-1 antitrypsin on PGD in male Yorkshire pigs, 30-35 kg. Donor lungs were injured through prolonged (24 hour) cold ischemic storage before transplantation. Recipient animals were randomized to receive either 500 mg/kg alpha-1 antitrypsin or saline placebo during the pneumonectomy before reperfusion, and every 24 hours thereafter until sacrifice at 72 hours (n=6 received alpha-1 antitrypsin treatment, n=6 received saline placebo). Treatment was administered intravenously, in a blinded fashion.

![Figure 4. Schema of ischemia-reperfusion injury model](image)

Lungs were retrieved from the donor animal following the lung retrieval procedure described above, and stored at 4°C for 24 hours. Recipient animals were allowed to acclimatize to the animal facility for one week prior to surgery. Fentanyl was administered through a skin patch 24 hours prior to surgery to manage pain. On the day of surgery, recipient animals were
sedated and anesthetized as described in the lung retrieval procedure. Intravenous cefazolin and methylprednisolone were administered, and saline replacement was continued intravenously throughout the procedure at 70-100 ml/h. The pig was intubated and ventilated in a protective manner. The neck was opened and dissected to place IV lines into the carotid artery and jugular vein. The lines were kept in place throughout the rest of the study period until sacrifice; the arterial line was used for blood pressure monitoring during the procedure and sampling during the 72 hours following transplantation, and the venous line was used for medication administration in the 72 hours following transplantation. A left thoracotomy was then performed. The left azygos vein, left pulmonary artery, and pulmonary veins were dissected, and heparin (10,000 IU) was administered intravenously. The left main bronchus was dissected and clamped. The left donor lung was dissected and prepared on the back-table, and then brought to the operating table. The bronchial anastomosis was performed, followed by pulmonary artery anastomosis, then atrial anastomosis. At this point, a second dose of heparin was administered to the recipient. The bronchial anastomosis was examined bronchoscopically; the lung was re-inflated and any secretions in the bronchus were suctioned. Finally, the lung was reperfused by gradually opening the pulmonary artery clamp. For post-operative management, a chest tube was inserted into the intercostal space, an intercostal block was administered (5ml bupivacaine (0.5%) and 5ml xylocaine (1%)), and the chest was closed. The pig was turned to the prone position and given buprenorphine (intramuscularly, 3 ml). Anesthesia was weaned, and the pig was extubated once it began to cough and given oxygen through a face mask as necessary.

Recipient animals recovered in the animal facility. They were monitored daily for signs of lethargy and appetite. Pain was treated with buprenorphine (0.01-0.05 mg/kg intravenously every 6 hours), and famotidine was administered twice daily (20 mg). The pigs were continuously immunosuppressed with methylprednisolone (1 mg/kg/day, intravenous) and cyclosporine (10 mg/kg/day orally). Enoxeparin was administered to prevent thrombo-embolisms, and ciprofloxacin and ceftazidime were administered as anti-microbials.

At sacrifice, animals were sedated and prepared as described in the donor procedure. Bronchoscopic evaluation of the implanted lung and anastomosis was performed. Lung compliance was assessed using the ventilator (Figure 3a). Pulmonary blood was sampled from the pulmonary vein and blood-gas analysis was applied to ascertain oxygenation (Figure 3b). The right hilum was clamped, and a systemic blood sample was taken from the arterial line to assess
graft oxygenation (*Figure 3c*). A BAL sample was obtained from both the graft and native lung: the bronchoscope was wedged as distally as possible in the airway, followed by two 10 mL injections of saline. The fluid was collected using a specimen trap, centrifuged at 3200 g for 10 minutes, and the supernatant was stored at -80° C until use. Finally, the double-lung block was excised and tissue was sampled.

NETs were analyzed in the lungs at 72 hours post-reperfusion by assessing the absorbance readings of NE-DNA complexes quantified by ELISA (see below) in the BAL samples. In a separate previous study, the same model was followed but with a lower dose of alpha-1 antitrypsin (240 mg/kg) and only 4 hours of reperfusion before sacrifice (Iskender, Sakamoto et al. 2016). BAL samples taken at the time of sacrifice were analyzed for NETs to compare with the BAL samples from 72 hours after reperfusion. Tissue biopsies were taken at the time of sacrifice and homogenized according to the protocol described below.

**NE-DNA ELISA**

All swine samples were analyzed for NE-DNA complexes using the same ELISA protocol. 100µL of undiluted samples were added in duplicate to a 96-well plate, which was pre-coated with anti-neutrophil elastase antibodies (MyBioSource, catalog #MBS269576). The plate was sealed and incubated for 90 minutes at 37° C. Wells were washed thrice with 350µL phosphate buffered saline, and 100µL of anti-DNA antibody was added (diluted 1:100 in incubation buffer; Roche, catalog #11774425001). The plate was sealed. After incubation at room temperature for two hours on a rocker, wells were washed again as before and 100µL of ABTS substrate (MyBioSource, catalog #MBS269576) was added to each well. After 30 minutes of incubation in the dark at room temperature, stop solution was added and the absorbance was read at 405 nm using a plate reader.

**Results**

NETs were detected in BAL samples taken from the left transplanted lung at the time of sacrifice (72 hours after reperfusion), but there was no significant difference between the alpha-1 antitrypsin treatment or saline placebo groups (p=0.4848, n=6 per group).
NETs in BAL samples 72 hours post-transplant in alpha-1 antitrypsin treated and control pigs. NETs, reported as OD of NE-DNA complexes, did not differ between animals treated with alpha-1 antitrypsin and those who received saline placebo in BAL fluid samples 72 hours post-transplant (p=0.4848, n=6 per group).

Figure 5. NETs in BAL samples 72 hours post-transplant in alpha-1 antitrypsin treated and control pigs

Lung function parameters did not vary significantly between treatment and control groups at 72 hours (Figure 3). Mann-Whitney analysis demonstrated a non-significant difference in static compliance between alpha-1 antitrypsin treatment and control groups (p=0.4567). Similarly, there was no significant difference in systemic arterial oxygenation after clamping of the right (native) hilum (p=0.3939), nor in left (graft) pulmonary vein oxygenation (p=0.3095).
Lung function of alpha-1 antitrypsin treated and control pigs 72 hours post-transplant. Lung function parameters did not differ significantly between pigs treated with alpha-1 antitrypsin or saline placebo 72 hours after single lung transplantation. Specifically, there was no significant difference between groups in lung static compliance ($p=0.4567, n=6$ per group) (a), oxygenation of the pulmonary vein ($p=0.2403, n=6$ per group) (b), and oxygenation from the transplanted lung ($p=0.3095, n=6$ per group) (c).

Figure 6. Lung function of alpha-1 antitrypsin treated and control pigs 72 hours post-transplant

NETs measured in BAL samples taken 4 hours after reperfusion in a study testing the effect of a 240 ml/kg dose of alpha-1 antitrypsin were significantly different between groups. NETs in the BAL of the treatment group ($n=5$) were significantly higher than NETs in the control group ($n=5$) ($p=0.0079$).
**NETs in BAL samples 4 hours post-transplant in alpha-1 antitrypsin treated and control pigs.** NETs, reported as OD of NE-DNA complexes, were measured in BAL samples taken 4 hours after reperfusion in a swine single lung transplant model. NETs were significantly higher in the alpha-1 antitrypsin treatment group, which received 240 ml/kg of intravenous alpha-1 antitrypsin (n=5), compared to the control group (n=5) (p=0.0079).

*Figure 7. NETs in BAL samples 4 hours post-transplant in alpha-1 antitrypsin treated and control pigs*

**Discussion**

This study investigated whether a large animal model of lung transplantation is relevant to the study of NETosis, whether NETs form in a swine model of lung ischemia reperfusion injury and how NETs are related to the degree and type of this injury, and whether the anti-inflammatory properties of alpha-1 antitrypsin affect NETosis in the context of lung transplantation. This study compared the concentration of NETs in BAL fluid samples from pigs 72 hours after lung transplantation. The lungs were subjected to prolonged cold ischemia (24 hours) prior to transplantation in order to induce injury, and recipient animals were randomized to receive either alpha-1 antitrypsin treatment or saline placebo in a blinded, randomized manner. The postulation guiding this study was that pigs who received alpha-1 antitrypsin treatment would have less lung injury and less NETs in the lungs, as measured in the BAL at the time of sacrifice. However, there was no discernible difference in the amount of NETs in the BAL samples taken from the alpha-1 antitrypsin-treated pigs and those in the control group.

The detection of NETs in BAL samples taken 72 hours after lung transplantation support the idea that large animal models are relevant platforms with which to study NETosis in lung injury, and that NETs form as a result of lung injury in these animal models. The lack of difference between alpha-1 antitrypsin treatment and control groups in BAL NET levels contradicts the initial prediction that alpha-1 antitrypsin would exert anti-inflammatory effects by suppressing NETosis. However, when considering the broader postulation that NETs detected in the lungs reflect the degree of lung injury, these results are not entirely surprising: pigs in both the treatment and control group demonstrated similar lung function at 72 hours after transplantation. The results of this three-day swine lung transplantation survival study contrast those of Sayah et al., who demonstrated that NETs form as a result of ischemia-reperfusion injury after hilar clamping as well as orthotopic lung transplantation in mice (Sayah, Mallavia et al. 2015). However, in this study, all pigs were healthy and demonstrated good lung function at 72 hours (for instance, the systemic PaO$_2$/FiO$_2$...
ratio was greater than 300 in all animals), whereas the mice subjected to prolonged cold ischemic time followed by transplantation in the study by Sayah et al. demonstrated worsened lung function at the time of NET analysis. The study by Sayah et al. also demonstrated a difference in NETs in BAL from patients who developed grade 3 PGD and no PGD; in the present study, while all pigs underwent the same process of ischemia-reperfusion injury, none of them developed PGD as it is clinically defined, due to the single lung transplant model and relatively good lung function throughout the 72 hours after transplantation. Therefore, while there was no difference observed in the NETs seen in the BAL of pigs treated with alpha-1 antitrypsin and treated with placebo, it is still possible that NETs are a reflection of injury in a swine ischemia-reperfusion model. Furthermore, alpha-1 antitrypsin may exert some anti-NETosis effect in a more injurious context, but this model of ischemia-reperfusion injury did not present the setting required to observe this effect.

There is also the possibility that NETs that formed as a result of ischemia-reperfusion injury were cleared or degraded well before 72 hours, and so were not detected in the lungs at the time of sacrifice in this study (Farrera and Fadeel 2013). For this reason, NETs in BAL samples taken 4 hours post-reperfusion were analyzed. These samples were from a separate study which shared the same lung injury model (24 hours of CIT) and treatment with alpha-1 antitrypsin, but differed in the alpha-1 antitrypsin dosage (240 mg/kg instead of 500 mg/kg) and time of sacrifice (4 hours after reperfusion instead of 72 hours). Lung injury and impaired function was apparent in the control group in this 4-hour survival model. Specifically, static compliance, pulmonary vein oxygenation (as measured by PaO$_2$/FiO$_2$ ratio), and arterial oxygenation from the graft alone were significantly superior in the alpha-1 antitrypsin treatment group, as compared to the control group (Iskender, Sakamoto et al. 2016).

Surprisingly, NETs were significantly higher in 4-hour BAL samples in the alpha-1 antitrypsin treatment group than in the control group (p=0.0079, n=5 per group). Higher levels of NETs in the alpha-1 antitrypsin-treatment group contradicted the prediction that alpha-1 antitrypsin would act to suppress NETosis in the context of ischemia-reperfusion injury due to its broad anti-inflammatory properties. Alpha-1 antitrypsin has been shown to initially amplify the inflammatory response before suppression, demonstrated through initial upregulation followed by suppression of myeloperoxidase and matrix metalloproteinase-9 after alpha-1 antitrypsin treatment (Koepke, Dresel et al. 2015). Thus, higher levels of NETs in the alpha-1 antitrypsin group at 4
hours after reperfusion may be a snapshot of this initial amplification process, and not reflect the full effect of alpha-1 antitrypsin treatment.

Another possible explanation relates to the different subpopulations of neutrophils. Sagiv et al. found that transforming growth factor-β mediates the conversion of high-density (“normal”) neutrophils to low-density neutrophils (Sagiv, Michaeli et al. 2015), and stimulates development of pro-tumor neutrophils rather than anti-tumor neutrophils (Fridlender, Sun et al. 2009). Alpha-1 antitrypsin has been found to inhibit the transforming growth factor-β pathway (Cho, Ryu et al. 2016); presumably, alpha-1 antitrypsin could therefore be having an effect on determining which neutrophil phenotype is present in the lungs, and thus predisposing neutrophils to undergo NETosis. This is currently speculation, but highlights the fact that the fields of NETosis and innate immune response to lung injury are rapidly evolving fields.

A limitation of this ischemia-reperfusion study is the lack of a healthy control group. As this study was initially designed to assess the effects of alpha-1 antitrypsin on ischemia-reperfusion injury and PGD, the two groups are the alpha-1 antitrypsin group and the placebo group. Due to concerns with the fragility of swine lungs, a baseline BAL sample prior to transplantation was not obtained. Therefore, while this study demonstrates the effect of alpha-1 antitrypsin on NETs in transplant recipients, it is difficult to assess the difference between NETs in the BAL of healthy pigs and transplanted pigs. Baseline BAL samples from other studies (i.e. NETs in aspiration injury) can be used as a rough estimate for a baseline NET level in the lungs, however these samples are not necessarily appropriate to include in the same analysis due to potential differences in BAL sample collection that could influence the detected concentration of NETs.

A further study was designed to assess the ability of neutrophils isolated from transplanted pigs in the alpha-1 antitrypsin study to undergo NETosis; the prediction being that neutrophils isolated from transplanted pigs treated with alpha-1 antitrypsin would demonstrate lower rates of NETosis compared to neutrophils from placebo-treated pigs. However, after preliminary results demonstrating a much lower response of control pig neutrophils to NETosis agonists, the study was redesigned to assess the difference in response of healthy control pig and control human neutrophils (further described in In vitro studies, below).
2.4 NETs in aspiration injury

**Aim 1:** to determine if the clinically relevant large animal (swine) model of lung transplantation and related lung injury was a suitable platform to study NETs

**Aim 2:** to test whether or not NETs form in clinically relevant lung injury models – specifically, donor lung injury, ischemia reperfusion injury, and severe aspiration injury – and assess their association with the degree or type of lung injury

**Aim 4:** to see if agents which have been described as anti-inflammatory or beneficial in the context of lung transplantation act by exerting a suppressive effect on NETs or NETosis

Methods

This study compared two groups: surfactant treatment or control. Pigs were randomly assigned to be treated with intrabronchial surfactant therapy, or a control recruitment manoeuvre (n=5 per group).

Pigs were anesthetized and tracheostomy was performed as previously described. Ventilation was initiated using pressure control of 15 cmH₂O peep of 5 and FiO₂ of 50%, respiratory rate was modified to maintain partial pressure of carbon dioxide (CO₂) of approximately 35-40 mmHg. Pigs were then cannulated for peripheral venous-venous ECMO using both femoral veins. Bronchoscopy was then performed and pre-injury BAL and plasma samples were taken. Gastric juice (pH of approximately 1.75) was then delivered to the lungs via bronchoscope to induce aspiration injury. Gastric contents were delivered in two installments separated by 15 minutes. After injury, a post-injury BAL and plasma sample were taken. ECMO was then initiated with a flow of approximately 1-1.7 L/min and an oxygen flow rate of 2 L/min. After 1 hour of ECMO, animals were either treated with intrabronchial surfactant (BLES®, surfactant proteins B and C) administration, or a control recruitment maneuver. After 5.5 hours, pigs were weaned off of ECMO by turning off the sweep. Lung function parameters (tidal volume/kilogram, pCO₂, PaO₂/FiO₂ ratio, static compliance) were recorded. A final BAL and plasma sample were taken. The animal was sacrificed 1 hour later.

NETs were quantified in the BAL and plasma from the three time points (baseline/before injury, after injury, after ECMO) using the ELISA described above. NETs in the different groups
at different time points were compared using a two-way ANOVA. Tissue from the time of sacrifice was homogenized (as described above), and NETs in tissue homogenate were compared between surfactant and control group using a Mann-Whitney analysis. Lung function parameters after ECMO were compared to NETs in the post-ECMO BAL sample using linear regression.

*Tissue homogenization*

Tissue biopsies were snap frozen in liquid nitrogen immediately after sample collection, and stored at -80°C until use. Tissue biopsies were manually ground using a mortar and pestle chilled on dry ice. Approximately 80 mg of ground tissue from each sample was transferred to a chilled centrifuge tube. Lysis buffer (10 mM HEPES buffer, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.6% Nonidet p-40, with one anti-protease cocktail tablet added just before use) was added to each centrifuge tube in a ratio of 1 mL per 50 mg of ground tissue. One stainless steel bead was added to each centrifuge tube. Centrifuge tubes were placed in a cooled TissueLyser II Adapter set (QIAGEN), and the TissueLyser II (QIAGEN) was run at 20 Hertz for 4 minutes. After 4 minutes, the Adapter set was rotated and the TissueLyser II was run again at 20 Hertz for another 4 minutes. The centrifuge tubes containing the now homogenized tissue samples were spun down in a pre-cooled (4°C) centrifuge at 12000 G for 10 minutes. The supernatants of the homogenized samples were collected, aliquoted, and stored at -80°C until analysis.

*Results*

The baseline BAL samples taken before injury showed similar low levels of NETs in both surfactant treatment and control groups. Samples taken immediately post-injury showed more variability in the levels of quantifiable NETs, and did not demonstrate a difference between groups. NETs showed the greatest variability in samples taken at the end of ECMO, but continued to not demonstrate any difference between surfactant treatment and control groups. Two-way ANOVA demonstrated that there was no significant interaction between treatment groups (surfactant or control), time points (baseline, post-injury, end of ECMO), and NETs in the BAL samples (p=0.9918). There was no statistical interaction observed between treatment groups (p=0.7732), but NETs did differ significantly in the BAL samples between different time points (p=0.0220).
NETs in plasma samples taken at the same time points (baseline, post-injury, end of ECMO) were virtually undetectable in both groups. Tissue homogenate from biopsies taken at the end of ECMO showed greater variability of NETs.

![Graph a) NETs in BAL](image)

![Graph b) NETs in systemic plasma](image)

![Graph c) NETs in tissue post-ECMO](image)
NETs in BAL, plasma, and tissue after weaning from ECMO in a severe swine aspiration injury model. In BAL fluid collected before injury, after injury, and after weaning of ECMO, NETs did not differ significantly between control and treatment groups (p=0.7732, n=5 per group) but did show a significant increase in both groups over time (p=0.0220) (a). NETs were virtually undetectable in plasma sampled at the same time points (b) and NETs did not demonstrate a significant difference in tissue homogenate post-ECMO (p=0.4206, n=5 per group) (c).

Figure 8. NETs in BAL, plasma, and tissue after weaning from ECMO in a severe swine aspiration injury model

When compared to lung function after ECMO was terminated (5 hours post-injury), NETs in the BAL demonstrated a positive linear relationship with PaO₂/FiO₂ ratio ($R^2=0.5094$, $p=0.0204$) as well as tidal volume per kilogram (TV/kg) ($R^2=0.4043$, $p=0.0481$). NETs showed a similar positive slope with static compliance at 5 hours, but this did not achieve statistical significance ($R^2=0.146$, $p=0.2759$). NETs showed a trend towards negative correlation with partial pressure of CO₂ at this time point ($R^2=0.3569$, $p=0.0682$).

NETs in BAL, plasma, and tissue after weaning from ECMO correlated with lung function parameters in a swine severe aspiration injury model. NETs were significantly positively correlated with PaO₂/FiO₂ ($R^2=0.5094$, $p=0.0204$, $n=10$) (a) and tidal volume/kg ($R^2=0.4043$, $p=0.0481$, $n=10$) (b). NETs and static compliance showed a positive slope but no statistically significant correlation ($R^2=0.146$, $p=0.2759$, $n=10$) (c) and NETs showed a trend towards negative correlation with CO₂ ($R^2=0.3569$, $p=0.0682$, $n=10$) (d).
Figure 9. NETs and lung function post-ECMO

Oxygenation as measured by PaO\textsubscript{2}/FiO\textsubscript{2} ratio was positively correlated with increasing tidal volume when both lung function parameters were analyzed using linear regression (R\textsuperscript{2}=0.8573, p=0.0001).

![Graph showing the relationship between tidal volume and PaO\textsubscript{2}/FiO\textsubscript{2} ratio post-ECMO](image)

**Figure 10. Relationship between post-ECMO tidal volume and oxygenation**

Discussion

This study investigated whether a swine model of lung aspiration injury, in the context of ECMO, is relevant to the study of NETosis. This study also examined whether NETs form in a swine model of lung aspiration injury and how NETs are related to the degree and type of this injury, and whether surfactant therapy, which has been described as beneficial for aspiration injury, affects NETosis in this setting.

NETs were detected in low levels in BAL fluid prior to injury (OD range: 0.10-0.26), suggesting some initial inflammatory response to the cannulation surgery. NETs increased in BAL samples after aspiration injury, and BAL NET levels were highest after the animals were weaned from ECMO, approximately 5 hours after the injury. These results indicate that this model is relevant to the study of NETosis in lung injury, and that NETs form as a result of insult to the lung in a swine model of lung aspiration injury. However, there was no significant difference between
NETs in surfactant treatment or control group BAL samples or post-ECMO tissue homogenate, suggesting that surfactant did not have a suppressive effect on NETosis.

Neutrophil-mediated response to acid instillation in the lungs peaks about 4 hours post-injury (Kennedy, Johnson et al. 1989). This is in line with what was observed in this ECMO gastric aspiration model regarding NETs: greater levels of NETs were detectable in BAL samples taken about 5 hours after injury was induced, compared to BAL samples taken just after the injury.

Surfactant proteins B and C contribute to the physiology of the lung by reducing surface tension at the air-liquid interface to promote proper lung function. Surfactant protein B has also been shown to have anti-inflammatory properties in the lung (Ikegami, Whitsett et al. 2005). Although surfactant protein D was not used in this study, it is worth noting that surfactant protein D has been shown to enhance the function of NETs with regard to bacterial trapping through binding to both the NETs and bacterial pathogens (Douda, Jackson et al. 2011). Although there was no difference in the quantity of NETs between the surfactant treated and control groups, it is possible that the administration of surfactant proteins B and C altered the functionality of NETs in a way that was not captured with these endpoints.

A major limitation to this study is the possibility of ECMO acting as a confounder. Although there is no difference in NETs between treatment and control group, it is difficult to elucidate whether the NETs observed at the end of ECMO can be attributed to the aspiration injury, ECMO, or both. As reviewed by Millar et al., ECMO is associated with a pro-inflammatory response which includes activation of neutrophils and platelets (Millar, Fanning et al. 2016). As NETosis is downstream of both neutrophil and platelet activation (Caudrillier, Kessenbrock et al. 2012), the NETs observed at the end of ECMO could be produced as a result of these ECMO-initiated inflammatory pathways.

Although it is impossible to know from this data the origin of NETosis in this model, it can be inferred that neither surfactant protein treatment nor ECMO helped to clear or decrease NETs in the alveoli within this timeframe. Of note, NETs were detected in the BAL as well as lung tissue homogenate from biopsies taken at the time of sacrifice. NETs were not detectable in plasma samples taken at any time point. The lack of NETs in systemic blood samples suggests that NETs are forming from neutrophils in the lungs, and are not entering the circulation in any measurable amount. The localization of NETs in the lung in this setting is supported by other reports of localized NETs in various types of lung injury (Sayah, Mallavia et al. 2015).
A guiding aim of this thesis is to determine whether NETs in the lungs reflect the severity of lung injury. Lung function at the end of ECMO was compared to NETs in BAL samples taken at this same time point. Linear regression of pulmonary function parameters and NETs in BAL samples demonstrated a significant association between PaO$_2$/FiO$_2$ ratio and NETs, as well as tidal volume per kilogram and NETs. There was a positive association with NETs and static compliance post-ECMO, but this association was not statistically significant. These results seem to indicate that NETs are associated with improved lung function. As NETs are described as inflammatory mediators, especially in the context of lung injury, it is initially surprising to find this positive association. All lungs in this study were severely injured; however, it is possible that NETs are forming in the less injured lungs in the study indicating that normal cell function and response to inflammation is occurring, while NETs do not form in the most injured lungs because the cellular responses are impaired.

Another possible explanation is that the increase in tidal volume is causing ventilator-induced lung injury, and NETs are forming in response to this. High tidal volume has been shown to induce NETosis in a mouse model (Yildiz, Palaniyar et al. 2015), and NETs are described as pathogenic in ventilator-induced lung injury (Li, Pan et al. 2017). It is possible that the observed corresponding increase in NETs and PaO$_2$/FiO$_2$ ratio in the study of NETs in aspiration injury and ECMO is, in fact, an artifact of the increase in tidal volume per kilogram. This increase in tidal volume could itself be a representation of ventilator-induced lung injury sustained by the animals throughout the course of the experiment. In this case, the correlation between NETs and increased tidal volume per kilogram as well as PaO$_2$/FiO$_2$ supports the idea that NETs are an indicator of the degree of lung injury; this idea is further supported by the findings of Li et al. that NETs in bronchial aspirates from patients who experienced gastric aspiration correlated with the severity of lung injury (Li, Zhou et al. 2018).

Examination of the lung function data shows that the animals who demonstrated the highest tidal volume per kilogram also demonstrated the highest PaO$_2$/FiO$_2$ ratios, and those who demonstrated lower tidal volumes were the same that showed lower PaO$_2$/FiO$_2$ ratios, further supporting this interpretation of the results (see Figure 7).
2.5 NETosis in experimental swine EVLP

**Aim 1:** to determine if the clinically relevant large animal (swine) model of lung transplantation and related lung injury was a suitable platform to study NETs

**Aim 2:** to test whether or not NETs form in clinically relevant lung injury models – specifically, donor lung injury, ischemia reperfusion injury, and severe aspiration injury – and assess their association with the degree or type of lung injury

**Aim 3:** to determine if EVLP is a suitable platform to study NETosis, by testing whether NETs can be detected in perfusate and whether detectable NETs could provide valuable information about the lungs

**Methods**

The same donor lung retrieval protocol was followed in all groups. Briefly, the pigs were sedated with ketamine (20 mg/kg, IM), midazolam (0.3 mg/kg, IM) and atropine (0.04 mg/kg, IV) prior to being transported to the operating room. Once in the operating room, pigs were initially anesthetized with inhaled isofluorane (3-5%) using a face mask, followed by intravenous administration of propofol (5-8 mg/kg/h) containing remifentanil (2-20 ug/kg/h). After performing a tracheostomy, the pig was ventilated (SERVO-i® Ventilator, Maquet Critical Care AB, Solna, Sweden) in a pressure-controlled manner: pressure control of 15 cmH₂O, PEEP of 5 cmH₂O, FiO₂ of 0.5 and respiratory rate of 15/min targeting tidal volumes of 6-8ml/kg, pO₂ greater than 200 mmHg, and pCO₂ of approximately 30 mmHg. The skin was cleaned with betadine and a thoraco-abdominal incision was made using a cautery pencil (Valleylab® Button Switch Pencil, Covidien, Mansfield, MA, USA). Access to the thorax was gained through a standard median sternotomy. The abdominal organs were removed from the body to alleviate pressure on the lungs prior to a sternotomy procedure. 10,000 IU of sodium heparin was injected intravenously, and the inferior vena cava, superior vena cava, aorta, and pulmonary artery (PA) were dissected. A cannula was inserted into the PA, the tip of the PA cannula was positioned before the PA bifurcation. A bolus of prostaglandin E₁ (500 mcg in 10 ml normal saline) was infused into the main pulmonary artery Aorta was cross-clamped. The superior vena cava was ligated. The inferior vena cava was
transected. The left atrium was transected at tip the left atrial appendage for drainage. Lung preservation solution, comprised of Perfadex® (XVIVO Perfusion, Denver, CO) with added THAM (0.6 ml/L), calcium (0.3 ml/L), and prostaglandin E1 (PGE₁) (500 mcg/3L) was used to flush the lungs in an anterograde fashion through the PA coming out from the heart. After excision of the heart, a retrograde flush of the lungs was performed using the same preservation solution via the left atrium and through the pulmonary veins. After flushing, the lungs were inflated, the trachea was clamped, and the double-lung block was excised and lungs were placed in an organ bag containing ice-cold Perfadex. The bag containing the lungs in Perfadex was placed on ice, and stored at 4° C until use.

Double-lung blocks were used for EVLP in all studies. The left atrium and pulmonary artery were cannulated (XVIVO). The EVLP circuit (XVIVO) was primed using acellular Steen® solution, and antibiotics (Cefazolin), immune system suppressants (Solu-medrol), and anticoagulants (Heparin) were added to the perfusate through the reservoir. The cannulated lungs were attached to the circuit, and perfusion was started following the Toronto protocol (see ‘Clinical Use of EVLP’ in the Literature Review, above). Lung perfusion was planned for 12 hours on the EVLP circuit, which is the standard length of time for experimental EVLP in our group.

The first model was a DCD model which included prolonged warm ischemic time. Cardiac arrest was induced by aortic clamping, followed by two hours of warm ischemic time. The lungs were then retrieved and stored on ice for 6 hours, prior to cannulation and ex vivo perfusion as described above. The second model was a prolonged cold ischemic time model. This model included donor lung retrieval and cold storage of the lungs in Perfadex® on ice for 24 hours, prior to cannulation. The third model was minimal cold ischemic time (cold ischemic time of less than two hours), which included retrieval of donor lungs, storage on ice for less than two hours, followed by cannulation and perfusion.

<table>
<thead>
<tr>
<th>Lung injury model</th>
<th>Ischemic time</th>
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<tbody>
<tr>
<td>DCD</td>
<td>2 h warm + 6 h cold</td>
</tr>
<tr>
<td>Prolonged CIT</td>
<td>24 h cold</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;2 h cold</td>
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Table 2. Lung injury models in swine EVLP

Perfusate samples were analyzed for up to 9 hours, and tissue biopsies obtained before EVLP and at the termination of EVLP (after 9-12 hours of perfusion) in the DCD and prolonged CIT groups were homogenized (protocol described above) and analyzed. All samples were analyzed for NETs using a sandwich ELISA test for NE-DNA complexes (protocol described above). Repeated measures analysis of variance (ANOVA) testing with Tukey’s post-hoc analysis was used to compare the levels of NETs in the perfusate between the different injury groups over the course of perfusion, and Wilcoxon matched-pairs analysis was applied to tissue biopsies taken before and after EVLP within the same groups.

Lung function was assessed throughout perfusion by measuring oxygenation, dynamic compliance of the lung, and pulmonary vascular resistance. Oxygenation was assessed throughout perfusion of all EVLP cases by measuring the ratio of arterial oxygen partial pressure to fraction of inspired oxygen (PaO₂/FiO₂ ratio). Pulmonary vascular resistance (PVR) was measured using the following calculation:

\[
PVR = \frac{(\text{Pulmonary arterial pressure} - \text{left atrial pressure})}{\text{Flow}} \times 80
\]

*Equation 1. Pulmonary vascular resistance*

Lung function was compared between different injury models at 1 hour, 5 hours, and 9 hours of perfusion using Kruskal-Wallis analysis.

Results

NETs were detected in the EVLP perfusate as well as lung tissue homogenate. In the perfusate, NETs in all lung injury model groups increased from the initial measurement after one hour of perfusion, over the course of nine hours. A two-way ANOVA test was utilized to assess the effects of different injury models and time points (e.g., time since beginning of perfusion) on the levels of NETs detected in the perfusate. The interaction between injury type groups and time since beginning of perfusion was insignificant (p=0.6015). However, injury type exerted a significant effect on NETs in the perfusate (p=0.0189), as did time since beginning of perfusion.
Tukey’s post-hoc analysis for multiple comparisons demonstrated that all injury types showed a significant increase in perfusate NETs from 1 hour of perfusion to 9 hours (control: \( p=0.0064 \); prolonged CIT: \( p=0.0003 \); DCD: \( p=0.0019 \)). Only the prolonged CIT group showed a significant increase in perfusate NETs from 1 hour to 5 hours (\( p=0.0027 \)), and increases from 5 hours to 9 hours were insignificant for all injury types.

**NETs increase over time and vary by ischemic donor injury in swine EVLP perfusate.** NETs were measured in EVLP perfusate over the course of perfusion, and were analyzed according to injury group as well as time point. NETs are reported as OD of NE-DNA complexes. Two-way ANOVA demonstrated a significant interaction between injury type and NETs in the perfusate (\( p=0.0189 \)) and between time since beginning of perfusion and NETs in the perfusate (\( p<0.0001 \)). NETs significantly increased between 1 hour and 9 hours in all injury groups (control: \( p=0.0064 \); prolonged CIT: \( p=0.0003 \); DCD: \( p=0.0019 \); \( n=5 \) per group); NETs significantly increased between 1 hour and 5 hours in the prolonged CIT group (\( p=0.0027 \), \( n=5 \)).

*Figure 11. NETs in swine EVLP perfusate*

Tissue homogenate from DCD tissue biopsies taken before and after EVLP showed a significant decrease in NET levels (\( p=0.0079 \)); tissue biopsies from the prolonged CIT group showed no difference in NET levels after EVLP (\( p=0.3095 \)).
NETs decrease in swine lung tissue homogenate after EVLP in a DCD and prolonged warm ischemia model. Lung tissue biopsies were taken before and after EVLP in the DCD and prolonged CIT groups. Biopsies were homogenized and NETs were detected with ELISA, reported as OD of the NE-DNA complexes. Wilcoxon matched pairs analysis showed that NETs decreased significantly after EVLP in DCD tissue homogenate (p=0.0079, n=5) (a), but showed no statistically significant difference after EVLP in prolonged CIT tissue (p=0.3095, n=5) (b).

All lungs in the control group (n=5) and prolonged CIT group (n=5) were perfused for at least 12 hours, which is the standard length of perfusion time for experimental EVLP in our center. However, three of the EVLP experiments in the DCD group were terminated between 9 and 11 hours due to extremely poor lung function (n=3); only 2 out of 5 sets of bilateral lungs were able to be perfused for 12 hours.

Lung function parameters varied between the different lung injury models at corresponding time points (Figure 10). The delta pO2, a measure of oxygenation of the lung, was significantly higher in the DCD group than the control group at 1 hour of perfusion (p=0.0056), although no difference was seen between the DCD and prolonged CIT group or between the prolonged CIT and control group (p=0.1431 and p=0.7737, respectively). Similarly, at 5 hours of perfusion, the DCD lungs were better oxygenated than control lungs (p=0.0279), but there was no statistical difference in the prolonged CIT group and neither DCD nor control groups (p=0.0509, p>0.9999, respectively). At 9 hours, there was no difference in oxygenation of any group (p>0.9999 for all groups). PVR was significantly higher in the DCD group compared to control.
group at 1 hour (p=0.0486), and this difference prevailed throughout the course of EVLP (p=0.0216 at 5 hours, p=0.0140 at 9 hours). While the prolonged CIT group did not differ from the DCD or control group at 1 hour (p>0.9999, p=0.0710, respectively), PVR was significantly higher in the prolonged CIT group than control group at 5 hours (p=0.0267) and 9 hours (p=0.040). There was no difference in the prolonged CIT and DCD groups at 5 or 9 hours (p>0.9999 for both time points). At 1 hour, there was no difference in dynamic compliance between groups (p=0.3079 for DCD vs. prolonged CIT, p=0.2281 for DCD vs. control, p>0.9999 for prolonged CIT vs. control). However, at 5 hours, while there was no difference between prolonged CIT lungs and neither control nor DCD lungs (p=0.0839 and p>0.9999 respectively), the control group demonstrated significantly higher compliance than the DCD group (p=0.0153). At 9 hours, the control group demonstrated significantly better compliance than either of the injury models (p=0.0265 DCD vs. control, p=0.0215 prolonged CIT vs. control), while there was no difference in compliance between the DCD or prolonged CIT groups (p>0.9999).
Lung function parameters in swine EVLP were worse in more severely injured lungs. Lungs sustained three different models of ischemic donor injury (n=5 per group) and were perfused on the EVLP circuit. Oxygenation during EVLP was significantly higher in the DCD group than the control group at 1 hour and 5 hours (p=0.0056 at 1 hour, p=0.0279 at 5 hours), but no difference was observed at 9 hours (a). Pulmonary vascular resistance was significantly higher in the DCD than control group at 1 hour (p=0.0486), and in the DCD and prolonged CIT groups compared to control at 5 hours (p=0.0216, p=0.0267) and 9 hours (p=0.014, p=0.040) (b). Dynamic compliance did not differ significantly at 1 hour, but was significantly lower in DCD than control at 5 h (p=0.0153) and in both DCD and prolonged CIT at 9 hours (p=0.0265, p=0.0215) (c).

**Figure 13. Lung function parameters during swine EVLP.**

Discussion

This study investigated whether a swine model of donor lung injury and subsequent EVLP is relevant to the study of NETosis, whether NETs form as a result of donor lung injury in a way that reflects the degree and severity of the injury, and whether EVLP can be a useful platform for the study of NETosis in lung transplant related injury.

This study compared NETs detected in EVLP of different donor lung injury models, as well as the lung function at associated time points. The DCD model demonstrated the poorest lung function, and the control group retained the best lung function throughout the course of perfusion. NETs in EVLP perfusate increased over time in all groups; however, NETs in perfusate were significantly associated with the donor lung injury group. NETs in tissue homogenate decreased after EVLP in the DCD group, but not in the prolonged CIT group.

This is the first description of NETs in EVLP perfusate, which importantly suggests that EVLP is a meaningful platform to study NETosis in lung transplantation. The results from the swine EVLP study demonstrate that NETs increase significantly in EVLP perfusate over time. This finding suggests that NETs are either produced in the donor lungs prior to EVLP, and continuously...
washed out of the lungs to become increasingly concentrated in the perfusate; or, neutrophils remain in the donor lungs and undergo NETosis throughout the course of EVLP. It is also possible that both of these scenarios are correct, and the primary mode of NET accumulation in EVLP perfusate depends on the type of injury that has occurred in the donor lungs.

A strength of this study design is the ability to compare NETs as downstream effects of different donor lung injury within the same platform (i.e. EVLP). Different donor injury groups showed a significant interaction with NETs in the perfusate, suggesting that the type or severity of injury influences the NETs in the perfusate. The DCD group was the most severely injured, shown by the poor lung compliance, high PVR throughout the nine hours of perfusion, and that only 2 out of 5 lungs in this group successfully completed 12 hours of EVLP. Interestingly, the DCD group showed higher perfusate levels of NETs at the beginning of perfusion than either of the other groups, and lung tissue homogenate from the DCD group demonstrated a significant decrease in NET levels after EVLP. This suggests that EVLP removed NETs from the lung tissue—presumably into the perfusate, as the levels of NETs rose throughout perfusion. In this case, it is plausible that EVLP performed a “wash-out” of the lungs with regard to NETs: NETs may have formed as a result of the severe lung injury inflicted in the donor lungs before perfusion (i.e. 2 hours of warm ischemic time), and been washed out into the EVLP circuit once perfusion began.

Meanwhile, the prolonged CIT lungs underwent relatively minimal injury at the time of death due to their static cold preservation immediately after retrieval. Thus, it is possible that while the NETs detected in the DCD lung EVLP were artifacts of previous injury (their increase in perfusate and simultaneous decrease in tissue represent a mechanical wash-out effect of perfusion), NETs detected in prolonged CIT lung EVLP perfusate represent active production of NETs as a part of the reperfusion process and ischemia-reperfusion injury during EVLP. This could explain why the DCD perfusate showed initially higher levels of perfusate NETs: the NETs detected at this time were the “wash-out” from the injury, while NETs were just starting to be produced in the control and prolonged CIT groups and so reflected a lower concentration in the perfusate. Furthermore, the prolonged CIT model of EVLP demonstrated an increase in perfusate NETs but no decrease in tissue NETs, indicating that NETs were not being removed from the lung through perfusion and thus could reflecting continuous production, perhaps due to the metabolic activation of reperfusion in the lung. This model was not a true “brain death” model; therefore, this offers insight into the
effects of EVLP on a cold preserved lung without the noise from a catecholamine or cytokine “storm”.

Active production of NETs could also explain why NETs increased, albeit to a much lesser degree, in the control group perfusate: the minimal CIT prevented the more severe injury observed in the prolonged CIT lungs (i.e. higher PVR at 5 and 9 hours; lower compliance at 9 hours), but NETosis may still have occurred in lungs on EVLP as a part of reperfusion. The fact that the prolonged CIT and control group animals underwent the same mode of death and only varied in the length of ischemic time, and the prolonged CIT group had higher NETs than the control group throughout the course of perfusion, indicates that NETs may be a marker of severity of lung injury.
2.6 Conclusions

Three studies were conducted to determine the presence of NETs in animal models of transplant-related lung injury. Firstly, NETs were assessed in donor lungs by sampling EVLP perfusate over the course of perfusion, as well as lung tissue biopsies before and after EVLP. The comparison of different lung injury models within this study demonstrated that NETs increase in perfusate over time, but the concentration of NETs in perfusate varies according to lung injury. In a 72-hour survival model of ischemia-reperfusion injury, NETs were detected in the lungs of recipient animals at the time of sacrifice. There was no difference in NETs at 72 hours post-reperfusion between animals who received treatment with the anti-inflammatory protein alpha-1 antitrypsin and those who received saline placebo; however, in a similar model, NETs in the lungs 4 hours post-reperfusion were significantly increased in the alpha-1 antitrypsin treatment group than the control group. Finally, NETs were assessed in animals subjected to aspiration injury and ECMO, and were shown to increase after the injury and course of ECMO. Surfactant therapy did not affect the levels of NETs in the lungs. Overall, these results demonstrate that NE-DNA complexes, representative of NETs, are detectable in multiple models of transplant-related lung injury in vivo.

In vitro, NETosis assays of pig and human neutrophils demonstrated different responses to well-described NETosis agonists. Therefore, in order to provide clinical relevance to what was learned about NETs in large animal models of lung transplantation, studies assessing NETs in clinical lung transplantation were designed. With the knowledge that NETs are detectable in EVLP perfusate and are produced in a wide variety of lung injury models, but are not reliably produced using conventional methods in isolated pig neutrophils, a focus on exploring the role of NETs in clinical lung transplantation was imperative.
Chapter 3: Human Studies
3.1 Rationale, Hypotheses, and Research Aims

Animal studies are important to the contribution of knowledge of NETs in lung transplant related injury; however, in order to fully understand the role of NETs in clinical lung transplantation, it is imperative that studies are designed based on clinical data and samples. The following studies were designed to contribute to the overarching hypothesis of this thesis, that NETs are important markers and effectors of lung transplant related injury. Specifically, the following studies were designed to examine whether what was learned from the swine models of lung transplant related injuries could be translated to clinical scenarios.

The first study in this series of human studies expanded on Chapter 2: Section 2.4, which aimed to determine if EVLP is a suitable platform to study NETosis, by testing whether NETs can be detected in perfusate and whether detectable NETs could provide valuable information about the lungs. The present study examined NETs in clinical EVLP perfusate samples in order to ascertain whether NETs could be detected in clinical EVLP samples. As NETs have not yet been described in EVLP perfusate, their detection in this setting is novel and important to the understanding of NETosis in lung transplantation.

Furthermore, this study assessed the utility of NETs in EVLP perfusate as a biomarker of clinical lung injury in transplant recipients. This conjecture was formulated from the premise that NETs in EVLP perfusate are a reflection of the degree and type of injury sustained by the donor lung, which was suggested from the results of the previously described swine study of donor lung injury and EVLP (in Chapter 2: Section 2.4). As such, NETs in EVLP perfusate could serve as a clinically relevant biomarker of lung injury.

EVLP offers a unique opportunity for assessment of organ function prior to transplantation, and the analysis of biomarkers in the donor lung via the EVLP perfusate offers a means of assessing the precise biological mechanisms occurring in the lung. A number of studies have addressed the presence of proteins such as pro-inflammatory cytokines in the donor lung as a predictor of recipient outcomes, through EVLP as well as donor lung BAL analysis (Fisher, Donnelly et al. 2001, Machuca, Cypel et al. 2015, Andreasson, Borthwick et al. 2017). As NETs are a product of inflammatory pathways, assessing the utility of NETs in the donor lung through EVLP perfusate as a predictor of recipient outcomes was a logical next study to translate what had been learned from the animal models of EVLP to a clinical setting.
Donor type, donor cause of death, donor age, donor BMI, and donor gender were included in the analysis to assess their potential influence on NETs in the donor lung. Brain death and cardiac death incite different cellular and molecular processes in the body, which can affect the lungs. As such, the type of donor could influence the NETs observed in donor lung perfusate. Specifically, DBD lungs were hypothesized to demonstrate higher levels of NETs in the perfusate than DCD lungs due to the intense inflammatory process that occurs during brain death. Donor cause of death was also included in the analysis, to elucidate specifically which mechanisms may be inciting NETosis in the lungs. Gender differences in immunity have also been described throughout the literature, and the observation that adult females tend to produce a higher inflammatory response than males (Klein and Flanagan 2016) led to the postulation that NETs in donor lung perfusate would be higher in females than in males. Donor BMI and age were analyzed to assess the clinical characteristics of the cohort and NETs.

NETs in donor lung perfusate were analyzed based on the outcome of EVLP, i.e. the decision to decline or accept the lungs for transplantation. This analysis aimed to determine if NETs in the donor lung were associated with the physician’s decision regarding the status of the lungs; while this decision involves assessment of lung function parameters as measured on EVLP, it is largely clinical. Therefore, association with levels of NETs could indicate that NETs are a useful, quantifiable measure of what is observed clinically.

In order to assess whether the NETs detected in clinical EVLP samples provide valuable and relevant information about the lungs, recipient characteristics indicative of lung function and patient status after transplant were chosen for analysis. NETs in lung transplant recipients have been described as pathogenic in PGD (Sayah, Mallavia et al. 2015). Therefore, NETs in donor lung perfusate were compared to recipient PGD development under the prediction that recipients of lungs with higher levels of perfusate NETs would be more likely to develop PGD. Similarly, higher levels of NETs in donor lung perfusate were hypothesized to be positively associated with recipient PaO\textsubscript{2}/FiO\textsubscript{2} ratio at the time of ICU arrival, number of days spent on the ventilator, and ICU length of stay.

The second and final study in this series aimed to further assess the clinical relevance of the findings from the large animal studies by examining NETs in human recipients after lung transplantation. This study aimed to assess the relevance of NETs in immediate and short-term
post-transplant outcomes. Understanding the role NETs play in recipients is essential to fully understand the role that NETs play in lung transplant related injury.

In order to understand the ramifications of NETs in the recipient, a study was designed to capture NETs in bronchial wash samples taken after reperfusion, and analyze these NETs in relation to recipient outcomes. Sayah et al. demonstrated that NETs are pathogenic in PGD and demonstrated higher NETs in BAL from transplant recipients that developed grade 3 PGD than grade 0 (Sayah, Mallavia et al. 2015). The aim of studying NETs in recipient bronchial wash was to expand upon the prior observed association of NETs and PGD development, by exploring the differences in donor characteristics as well as a variety of recipient outcomes in addition to PGD development. This study aimed to complement the study of NETs in the donor lung perfusate by examining the role of NETs in the recipient just after reperfusion, in order to illustrate a complete picture of the role of NETs in lung transplantation.

NETs in recipient bronchial wash were conjectured to be associated with degree of ischemia reperfusion injury, implying NETs would also be associated with subsequent PGD development and adverse recipient outcomes. NETs in the recipient lungs immediately after reperfusion may be produced as a result of ischemia-reperfusion induced NETosis. However, as NETs can propagate a cycle of neutrophil recruitment and NETosis, residual NETs in the donor lungs may contribute to an amplified NETosis response post-reperfusion in the recipient. Additionally, trends of NETs in swine EVLP perfusate differed according to donor type and severity of injury (see Chapter 2: Animal Studies). Therefore, this study assessed whether donor characteristics, such as type of donor (DCD or DBD) and use of EVLP, would affect the NETosis response in the recipient post-reperfusion.
3.2 NETs in clinical EVLP

**Aim 3:** to determine if EVLP is a suitable platform to study NETosis, by testing whether NETs can be detected in perfusate and whether detectable NETs could provide valuable information about the lungs

**Aim 6:** to translate the findings of the large animal studies to human studies to assess the clinical relevance

**Methods**

This study analyzed NETs in clinical EVLP perfusate samples obtained after four hours of perfusion. The perfusate samples were snap frozen in liquid nitrogen, and stored in the Toronto Lung Transplant Program Biobank at -80°C until use. Between 2008 and February 2017, 307 clinical EVLP cases occurred at Toronto General Hospital. There were 200 4-hour perfusate samples available for analysis from this time period, which were included in this study.

The same sandwich ELISA method that was used for the porcine samples was applied for the clinical samples, with minor modifications to the protocol as per manufacturer’s instructions. A 96-well plate pre-coated with anti-human polymorphonuclear leukocytes antibodies (Abcam, catalog #ab119553) was washed twice with 400 μL of wash buffer (Abcam, catalog #ab119553); the wash buffer soaked the wells for approximately 10 seconds between washes, and the plate was inverted and tapped to remove excess moisture after the last wash. 100 μL of perfusate samples, diluted 1:10 in phosphate buffered saline, was added to the wells in duplicate. The plate was sealed and incubated for 1 hour at room temperature on a rocker. After 1 hour, the plate was washed four times with 400 μL of phosphate buffered saline in each well. 100 μL of anti-DNA antibody, diluted 1:100 in incubation buffer (Roche, catalog #11774425001), was added to each well. The plate was sealed and incubated at room temperature on a rocker for 2 hours. After 2 hours, the plate was washed three times with 350 μL of phosphate buffered saline. 200 μL of tetramethylbenzidine (Abcam, catalog #ab119553) was added to the wells. The plate was incubated in the dark for approximately 20 minutes, until the color change reaction was complete. 50 μL of stop solution (Abcam, catalog #ab119553) was added to stop the reaction, and the wells were read using a spectrophotometer at 450 nM, with a 620 nM reference wavelength.
In order to standardize optical density readings across different plates, a positive control was created by isolating neutrophils from a healthy human control and stimulating the neutrophils with PMA for NETosis as described in Part I: Animal Studies. Sytox Green dye was added to two wells to confirm NETosis, and the remaining neutrophils were incubated without any dye. After four hours, the supernatant from the wells containing NETs was collected, combined, aliquoted, and frozen at -80°C until required for use in the ELISA (Cooper, Palmer et al. 2013). In addition, two samples were run on every plate to ensure consistency in the readings. The resulting optical densities were normalized to the positive control as well as the two repeated samples in order to compare and analyze ELISA results from different plates.

NETs measured in all samples were included in the analysis of donor characteristics (e.g. donor age, BMI, type, cause of death, and EVLP outcome). Recipients who were admitted to the ICU prior to transplantation were excluded from the analysis of NET and recipient outcomes (PaO₂/FiO₂ ratio at ICU arrival, PGD development, recipient days on the ventilator, recipient length of ICU stay). A summary of the sample cohorts is shown in Figure 14.

The association of donor characteristics and NET quantity in the perfusate was assessed using linear regression or Kruskal-Wallis testing in GraphPad Prism 7.0 (La Jolla, CA). These comparisons were made for the overall study cohort, as well as subgroups; Bonferroni correction was applied for multiple comparisons analysis. Linear regression and Kruskal-Wallis testing with Bonferroni correction were also used to assess recipient outcomes and NETs. The decision to accept or decline lungs for transplant and NETs was analyzed using a Mann-Whitney test. Alpha was set to 0.05 for all statistical analyses. A list of the analyzed parameters is available in Table 3.

<table>
<thead>
<tr>
<th>Donor characteristics</th>
<th>EVLP outcomes</th>
<th>Recipient outcomes</th>
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<tr>
<td>Donor type</td>
<td>Lung accepted or rejected for transplantation</td>
<td>PaO₂/FiO₂ at ICU arrival</td>
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<td>Cause of death</td>
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<td>Age</td>
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<td>Body Mass Index (BMI)</td>
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<td>Gender</td>
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*Table 3. Parameters included in EVLP perfusate NETs analysis*
In order to address concerns of a possible statistical outlier in the analysis of NETs and recipient days on the ventilator (discussed in detail in Results, below), a ‘Robust regression and Outlier removal’ tool was applied (Graphpad Prism 7.0 (La Jolla, CA)) to the optical density values representing NETs in the perfusate samples. The coefficient Q was set to 1%. Subsequent “clean” datasets were again analyzed using the same linear regression and Bonferroni correction as described above.

For this study, PGD grade at 72 hours was used for analysis. PGD was graded as per ISHLT guidelines. Briefly, a PaO₂/FiO₂ ratio greater than 300 mmHg and absence of radiographic infiltrates was awarded a PGD grade of 0; PaO₂/FiO₂ ratio greater than 300 mmHg and presence of infiltrates was awarded grade 1; PaO₂/FiO₂ ratio between 200-300 mmHg was awarded grade 2; and PaO₂/FiO₂ ratio less than 200 mmHg was a grade of 3. Patients were considered to have PGD 0 if they were extubated within the first 72 hours, and PGD 3 if ECMO was required within the first 72 hours.

Results

From the 4-hour perfusate samples included in this study (n=200), 111 were from DBD donors and 89 were from DCD donors. An approximately equal ratio of transplanted to declined lungs was found in both donor type groups (for DBD lungs, 69 lungs were transplanted and 42 were declined; within the DCD cohort, 59 lungs were transplanted and 30 were declined). A total of 13 recipients were excluded from analysis due to pre-transplant ICU admission (6 recipients in the DBD group and 7 in the DCD group). This resulted in 63 DBD recipients and 52 DCD recipients suitable for analysis.
NETs were higher in DBD lungs than DCD lungs, although this difference did not achieve statistical significance (p=0.0799). When stratified by donor cause of death, a Kruskal-Wallis analysis yielded a non-significant difference in median detected NETs ($\chi^2 = 7.658, p=0.0536$), with a mean rank of 51.09 for anoxia/cardiac arrest, 68.58 for cerebrovascular accident/stroke, 68.41 for head trauma, and 83.86 for unknown/other cause of death.

**Figure 14. EVLP perfusate study cohort**

NETs in EVLP perfusate do not significantly differ according to donor type or donor cause of death. Clinical EVLP perfusate was sampled at 4 hours of perfusion. NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. NETs in DBD lung perfusate tended to be higher than DCD perfusate, although this did not achieve statistical significance (p=0.0799, n=200) (a). NETs did not differ significantly based on causes of death (p=0.0536) (b).

**Figure 15. NETs in perfusate and donor types and donor causes of death**
There was no significant difference in the NETs detected in 4-hour perfusate samples between male or female donors for all donor types, or between donor types within female donors (p=1.000 for both). Within male donors, NETs were higher in DBD lungs than DCD, although this difference did not achieve statistical significance (p=0.0990). There was no significant correlation with donor age and NETs for all donors, or within donor types (p=1.000 for all donors and DBD donors; p=0.9333 for DCD donors). Within donor types, there was no significant correlation by gender for donor age and NETs (p=1.000 for DBD males, DBD females, DCD males; p=0.4734 for DCD females).

There was no significant correlation with perfusate NETs and overall donor BMI, or when stratified by donor type (p=1.000 overall and DBD donors; p=0.5952 for DCD). When stratified by gender within donor type, there was no significant correlation between BMI and perfusate NETs for either donor gender (p=1.000 for DBD males, DBD females, DCD males; p=0.5445 for DCD females).
NETs in clinical EVLP perfusate are not associated with donor age, gender, or BMI. Clinical EVLP perfusate was sampled at 4 hours of perfusion. NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. NETs did not differ significantly between male and female groups overall (p=1.000, n=200). NETs tended to be higher in male DBD perfusate than DCD perfusate (p=0.0990, n=126) and showed no difference between donor type within female donors (p=1.000, n=72) (a). There was no significant association between NETs and donor age within the overall cohort (p=1.000, n=200) or within DCD lungs (p=0.9333, n=89) or DBD lungs (p=1.000, n=111). Within donor types, there was no significant correlation by gender for donor age and NETs (p=1.000 for DBD males, DBD females, DCD males; p=0.4734 for DCD females) (b). There was no significant correlation with NETs and donor BMI in the overall cohort (p=1.000, n=200), nor the DBD (p=1.000) or DCD cohort (p=0.5952). When stratified by donor gender, there was no correlation between NETs and donor BMI (p=1.000 for DBD males, DBD females, DCD males; p=0.5445 for DCD females) (c).

Figure 16. NETs in perfusate and donor gender, age, and BMI.

NETs were higher in lungs that were ultimately declined for transplant than those that were accepted, in the overall cohort as well as within DCD lungs and DBD lungs; however, none of these comparisons achieved statistical significance (p=1.000 for all).

NETs in DCD males were higher in declined than accepted lungs, although this did not achieve statistical significance (p=0.9429). NETs in other donor and gender categories did not differ between declined or accepted lungs (p=1.000 for all).
NETs in donor lung EVLP perfusate are not associated with EVLP outcome. Clinical EVLP perfusate was sampled at 4 hours of perfusion. NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. NETs did not differ significantly between lungs that were accepted or declined for transplant in the overall cohort (p=1.000, n=200) (a). Similarly, NETs did not differ between EVLP outcomes when stratified by donor type (DCD: p=1.000, n=89; DBD: p=1.000, n=111) (b). NETs did not differ based on EVLP outcome within stratification of DCD males or females (males: p=0.9429, n=56; females: p=1.000, n=32) (c), or DBD males or females (males: p=1.000, n=70; females: p=1.000, n=40) (d).

Figure 17. NETs in perfusate and EVLP outcome

The PaO$_2$/FiO$_2$ ratio, an indicator of oxygenation in a patient, at the time of ICU arrival was not significantly associated with donor NETs in the overall study cohort ($R^2=0.002581$, p=1.000). Likewise, no association was found between PaO$_2$/FiO$_2$ ratio for DCD or DBD groups,
or gender types within those groups (DCD males: R²=0.001046, p=1.000; DCD females: R²=0.05939, p=1.000; DBD males: R²=0.04483, p=0.9025; DBD females: R²=0.02358, p=1.000).

NETs in EVLP perfusate are not associated with recipient oxygenation at the time of ICU arrival. Clinical EVLP perfusate was sampled at 4 hours of perfusion. NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. NETs in EVLP perfusate did not significantly associate with recipient PaO₂/FiO₂ ratio at the time of ICU arrival (R²=0.002581, p=1.000, n=113).

Figure 18. NETs in perfusate and PaO₂/FiO₂ ratio at ICU arrival

The majority of patients in the sample cohort did not develop PGD (n=85, PGD 0 or 1; n=13, PGD2; n=16, PGD 3; n=1 missing data and so excluded from analysis). Patients with a PGD grade of 0 or 1 were compared to patients with grade 3 PGD for the analysis. Mann-Whitney analysis demonstrated that patients who developed severe PGD received donor lungs with higher levels of NETs in the perfusate, although this difference did not achieve statistical significance (p=0.0745).
NETs in EVLP perfusate tend to be higher in perfusate of lungs which ultimately develop severe primary graft dysfunction. Clinical EVLP perfusate was sampled at 4 hours of perfusion. NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. NETs trended towards being higher in perfusate of lungs which ultimately developed severe primary graft dysfunction (PGD3) as compared to those that developed no primary graft dysfunction (PGD0) (p=0.0745, n=101).

*Figure 19. NETs in perfusate and PGD development*

NETs were significantly associated with recipient days on the ventilator ($R^2=0.1101$, p=0.0027). When analyzed by donor type, NETs in perfusate from DCD lungs demonstrated a significant association with recipient days on ventilator ($R^2=0.2472$, p=0.0018). NETs in DBD lung perfusate did not share this association with recipient days on ventilator ($R^2=0.01902$, p=1.0000). When stratified into male and female donors, NETs were significantly associated with recipient days on ventilator in female donor lungs ($R^2=0.2306$, p=0.0099), but not in male donor lungs ($R^2=0.02524$, p=1.0000). When stratified further into donor gender and type categories, DCD females demonstrated a strong association between NETs in the donor perfusate and recipient days on the ventilator ($R^2=0.5361$, p=0.0009); this association was not observed in DCD males ($R^2=0.002868$, p=1.0000) or in either gender in the DBD group ($R^2=0.08362$, p=0.5697 for DBD males; $R^2=0.07614$, p=1.0000 for DBD females).
NETs in EVLP perfusate are significantly associated with recipient days on the ventilator. Clinical EVLP perfusate was sampled at 4 hours of perfusion. NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. Linear regression analysis demonstrated that NETs were significantly associated with recipient ventilator days in the overall study cohort \(R^2=0.1101, p=0.0027, n=200\) (a). When stratified by donor type, DCD NETs were associated with ventilator days \(R^2=0.2472, p=0.0018, n=52\) (b) but DBD NETs were not \(R^2=0.01902, p=1.0000, n=63\) (b). NETs from female donors were significantly associated with ventilator days \(R^2=0.2306, p=0.0099, n=43\) (c) but not from male donors \(R^2=0.02524, p=1.0000, n=72\) (c). Within the DCD group, female donor NETs were associated with ventilator days \(R^2=0.5361, p=0.0009, n=22\) (c) while males were not \(R^2=0.002868, p=1.0000, n=30\) (d). In the DBD group, there was no association of either gender donor with recipient days on the ventilator (male: \(R^2=0.08362, p=0.5697, n=42\); female: \(R^2=0.07614, p=1.0000, n=21\) (e).

Figure 20. NETs in perfusate and recipient days on ventilator

Analysis of perfusate NETs and recipient length of ICU stay yielded similar results to the recipient days on ventilator. Overall, there was no significant association between NETs and the amount of days spent by the recipient in the ICU \(R^2=0.05885, p=0.0810\). Within donor types, recipients of DCD lungs demonstrated a significant association between NETs and ICU length of stay \(R^2=0.1704, p=0.0216\) than DBD lungs, which did not show any significance \(R^2=0.003096, p=1.0000\). NETs from male donors were not associated with recipient days in the ICU.
NETs in DCD and DCD female EVLP perfusate are significantly associated with recipient days in the ICU. Clinical EVLP perfusate was sampled at 4 hours of perfusion. NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. Linear regression analysis demonstrated that NETs showed a trend towards association with recipient days in the ICU overall study cohort ($R^2=0.05885$, $p=0.0810$, $n=200$) (a). When stratified by donor type, DCD NETs were associated with ICU days ($R^2=0.1704$, $p=0.0216$, $n=52$) but DBD NETs were not ($R^2=0.003096$, $p=1.0000$, $n=63$) (b). Donor gender stratification showed no significant association with ICU days in either female nor male donor NETs (female: $R^2=0.1413$, $p=0.1170$, $n=43$; male: $R^2=0.005855$, $p=1.0000$, $n=72$) (c). Within the DCD group, female donor NETs were associated with days in the ICU ($R^2=0.4238$, $p=0.0090$, $n=52$) while DCD males were not ($R^2=0.0004354$, $p=1.0000$). Neither DBD males nor females showed a significant association with recipient days of ICU (DBD males: $R^2=0.07154$, $p=0.7812$; DBD females: $R^2=0.1622$, $p=0.6318$).
In the DCD cohort, one sample from a female donor demonstrated very high NETs as well as days in the ICU and on the ventilator. There is no biological or clinical reason to exclude this sample from the analysis, but concerns were raised that this single patient may be skewing the data to support a significant association between NETs and recipient outcomes which may not truly exist. To investigate this concern, a Robust regression and Outlier removal method (Graphpad Prism 7.0; La Jolla, Ca) was applied to determine if this data point was a statistical outlier in the recipient days on ventilator and days in ICU results.

One statistical outlier was confirmed in the entire sample cohort as well as in the DCD cohort. However, there were no statistical outliers in the female donor cohort or DCD female cohort. When the statistical outlier was excluded from the linear regression analysis, there was no significant association between all samples NETs and recipient days on the ventilator ($R^2=0.01735$, $p=0.1624$) nor within the DCD group ($R^2=0.02118$, $p=0.3082$). Similarly, there was no significant association between all NETs and recipient days in the ICU ($R^2=0.003259$, $p=0.5463$) nor within the DCD group ($R^2=0.01253$, $p=0.4341$).
Exclusion of statistical outliers results in no significant association between NETs and recipient outcomes. Clinical EVLP perfusate was sampled at 4 hours of perfusion. NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. Linear regression was used to assess correlation between perfusate NETs and recipient ventilator days and days in ICU. When statistical outliers were excluded, there was no significant association within the entire study sample cohort or the DCD cohort between NETs and recipient days on ventilator (overall cohort: $R^2=0.01735$, $p=0.1624$, $n=114$; DCD: $R^2=0.02118$, $p=0.3082$, $n=51$) (a). Similarly, there was no significant association between NETs and recipient days in the ICU (overall: $R^2=0.003259$, $p=0.5463$, $n=114$; DCD: $R^2=0.01253$, $p=0.4341$, $n=51$) (b).

Figure 22. NETs in perfusate and recipient outcomes excluding statistical outliers

Discussion

This study aimed to elucidate whether what was learned from the large animal studies could be translated to humans, and thus whether NETs are detectable in clinical EVLP perfusate and if NETs in clinical perfusate provide valuable information about the lung. This study demonstrates, for the first time, that NETs can be detected in clinical EVLP perfusate. NETs were analyzed in EVLP perfusate with regard to donor characteristics as well as recipient outcomes. No significant differences or associations were found when donor age, body mass index, gender, cause of death, or type of donor were compared to quantity of detected NETs. NETs were also not significantly different between lungs that were ultimately accepted or declined for transplantation. NETs were not associated with development of primary graft dysfunction or PaO$_2$/FiO$_2$ ratio after transplant. However, NETs in donor lung EVLP perfusate were significantly associated with recipient days on the ventilator. Furthermore, this association was strengthened when the cohort was stratified by
donor type and gender, in the female and DCD groups. NETs were also significantly associated with recipient length of stay in the intensive care unit in the DCD and DCD female groups.

The detection of NETs in clinical EVLP perfusate is a novel finding. As discussed previously, EVLP offers a unique window to assess donor lungs prior to transplantation. The study of NETs in clinical EVLP perfusate attempted to elucidate some of the factors that may influence NETosis in donor lungs, and how NETs in donor lungs may affect or be associated with the transplant outcomes in the recipient. Examination of the predictive ability of NETs in the overall study cohort demonstrated that NETs in donor lung perfusate are associated with adverse recipient outcomes, such as recipient length of ICU stay or days on the ventilator. Examining NETs in smaller subgroups, such as female or DCD donors, decreased the statistical power of the analysis but offered more detailed insight into the biological factors at play.

NETs were not associated with the decision to accept or decline lungs for transplant after EVLP. Lungs are accepted or rejected for transplantation based on the transplant surgeon’s assessment of lung physiological function, such as compliance and oxygenation, as well as gross examination of the lung. This suggests that higher levels of NETs in the perfusate were not associated with the function or appearance of the lung within the first four hours of EVLP. However, since greater levels of perfusate NETs in DCD lungs were associated with longer recipient ICU stay and days on the ventilator, NETs are in this context potentially a more precise predictor of lung function post-transplant than the physiological assessment done during EVLP.

While the quantity of NETs did not differ in perfusate from DCD and DBD lungs, there was a clear difference in the predictive capacities of perfusate NETs in these different groups. Analysis of various donor and recipient parameters with regard to NETs generally only showed significant association in the DCD cohorts, and showed no association in the DBD cohorts. One possible reason for this is the biology of the lung injury that occurs during brain death and the associated catecholamine storm, which overwhelms the circulation with pro-inflammatory cytokines. It could be that this overload of inflammatory mediators creates a pro-inflammatory milieu which would make it difficult to delineate between NETs resulting from donor lung injury and NETs resulting from brain death-related cytokine release. However, biomarkers have successfully predicted adverse recipient results in donor lung tissue biopsies from a DBD cohort (Saito, Takahashi et al. 2013). Interestingly, another report of biomarkers in EVLP perfusate also only found significant associations in the DCD cohort (Machuca, Cypel et al. 2015). Therefore, it is possible that the ex
vivo perfusion incites a process of metabolic reawakening that unfolds differently in DCD and DBD lungs, potentially due to the pro-inflammatory state in which the DBD lungs are preserved.

Another possible explanation for this difference, which is also a limitation to the design of this study, is the fact that the current standard of care at Toronto General Hospital is to assess all DCD lungs by EVLP prior to transplant, but only assess marginal or high-risk DBD lungs on EVLP. This means that the DCD cohort is a more representative population of DCD lungs, while the DBD cohort only represents the most injured or worrisome lungs. By this token, assessing biomarkers in the two groups is not a completely fair comparison. It is for this reason that we analyzed donor and recipient parameters within each donor type group separately, to elucidate a more precise understanding of what is occurring in the donor lung. This could also explain why significant associations are found in the DCD group, but not the DBD group: the DBD group is already a relatively small subpopulation of the most injured lungs.

Notably, the trends seen in the swine EVLP models may shed some light on why there seems to be such a disparity in the trends seen in clinical perfusate analysis. In the swine models, the NETs increased in DCD perfusate and decreased in DCD tissue over time, suggesting a wash-out effect by the perfusion. Presumably, events that occur to the DCD donor, or during the warm ischemic time undergone by DCD lungs, may trigger inflammatory processes that can in turn incite NETosis in the lungs. In this case, NETs measured in the perfusate are a reflection of the degree of injury that has already occurred and are removed from the donor lungs through EVLP. The relationship between the amount of NETs in the perfusate and recipient outcomes, such as recipient days on the ventilator and in the ICU, supports this theory.

In addition to different trends in the DCD and DBD groups, NETs in perfusate from male donor lungs differed from NETs from female donor lungs. When the study cohort was analyzed by donor gender, the association of NETs in EVLP perfusate that was observed in the entire cohort was retained in NETs from female donors, but not in NETs from male donors. The body of literature surrounding gender differences in immunity strongly suggests that gender differences exist in NETosis, especially due to the influence of hormones such as estrogen and progesterone (Giaglis, Stoikou et al. 2016). The observations in the present study support this suggestion, as associations were seen between female donor NETs and recipient outcomes, but not male donor NETs.
Analysis for potential ‘outliers’ was important to do for this study, as there were concerns that a single data point was skewing the data to support an association between NETs and recipient days on the ventilator and in the ICU which did not truly exist. Again, there was no biological or clinical reason to exclude this data point, but this exploration of the analysis was done to determine the strength of the results. While one ‘outlier’ was identified in the entire study cohort and in the DCD cohort, there was no statistical ‘outlier’ identified in the female or DCD female cohorts. This suggests that the relationship between NETs and recipient outcomes is stronger in female donor lungs – specifically, DCD female lungs – than in DBD or male donor lungs. These results imply that both gender and donor type differ biologically, and that these categorizations are essential to take into consideration when attempting to define a biomarker of lung injury or predictor of lung transplant recipient outcomes.

As PGD is the leading cause of morbidity and mortality after lung transplantation, the relationship of NETs in donor lung perfusate and corresponding recipient development of PGD was analyzed. Although not significant, there was an observed trend of higher NETs in the perfusate of lungs received by patients who ultimately developed PGD. These results, although not statistically significant, support the prediction that higher levels of NETs in the donor lung perfusate would be associated with PGD development in the recipient.

Both recipient ICU length of stay and recipient days on the ventilator were examined, since they offer important criteria to determine the recipient short-term quality of life as well as burden on hospital resources. The results of the analysis of these parameters and perfusate NETs were similar, which is unsurprising: to be on the ventilator, a patient must be in the ICU and so there is a great deal of overlap in the results. The days on the ventilator may offer a more precise measurement because this is directly related to the recipient’s ability to breathe through the grafted lung; however, both parameters are important to consider as lung transplantation is a complex process and complications not necessarily directly related to the graft function, such as cardiac or neurologic function, can impact the recipient quality of life. As NETs have both beneficial and detrimental roles in the immune system, it was important to see how they were associated with the care required for recipients immediately post-transplant.
3.3 NETs in recipient bronchial wash post-reperfusion

**Aim 6:** to translate the findings of the large animal studies to human studies to assess the clinical relevance

**Methods**

As a part of routine protocol, the endotracheal tube in the transplant recipient is changed before the patient is transferred to the ICU. At this time, a bronchial wash is performed. These samples were collected from the study cohort (n=37) and stored at 4°C temporarily. Samples were then processed by centrifugation for 10 minutes at 4000 rpm (3220 g) with an Eppendorf centrifuge 5810R. The supernatants were collected and stored at -80°C until analysis. NETs were measured using the same NE-DNA ELISA technique described above for the clinical EVLP perfusate samples, with a 1:10 dilution of the BW samples in sample diluent (Abcam, catalog #ab119553). Results are reported as relative optical density as measured with a spectrophotometer.

NETs in these bronchial wash samples were analyzed for associations with donor type, donor gender, recipient PGD development, recipient PaO₂/FiO₂ at the time of ICU arrival, recipient days on the ventilator, and recipient days in the ICU. One sample (n=1) was excluded from analysis as the absorbance was out of range for detection. These analyses aimed to determine both the potential influence of donor characteristics and the effects of EVLP with regard to NETs on recipient outcomes. The same statistical methods were used for this study as were described for the previous study (*I. NETs in clinical EVLP perfusate*, above). A summary of the sample cohorts is shown in *Figure 23.*

**Results**

Samples were collected from 37 patients, most of whom received lungs from DBD donors (n=30; n=6 DCD lungs). In the analyzed cohort, the majority of lungs were transplanted without the use of EVLP (n=25). All DCD lungs underwent EVLP prior to transplantation (n=6), and the minority of DBD lungs underwent EVLP (n=6). All samples were analyzed for donor characteristics (use of EVLP, donor type); however, recipients in the ICU prior to transplantation
were excluded from the analysis of recipient outcomes (n=3 excluded). A summary of the sample cohort is shown in Figure 23, below.

![Bronchial wash NETs study cohort](image)

There was no significant difference detected in NET levels in recipient bronchial wash samples between recipients who received lungs from DBD or DCD donors (p=0.6942), nor was there a difference between EVLP and non-EVLP groups (p=0.4156). Similarly, no difference was seen in bronchial wash NETs between male and female recipients (p=0.5757).
NETs in recipient bronchial wash do not associate with EVLP, donor type, or recipient gender.
Routine recipient bronchial wash samples were collected just before recipients were transported to the ICU (n=36). NETs were measured in these samples using ELISA for NE-DNA complexes, and are reported as OD. NETs did not differ in bronchial wash samples when analyzed by use of EVLP (p=0.4156) (a), donor type of death (p=0.6942) (b), or recipient gender (p=0.5757) (c).

**Figure 24. Effect of EVLP, donor type, donor and recipient genders on NETs in recipient bronchial wash**

Linear regression analysis of NETs in bronchial wash samples and recipient outcomes yielded no significant associations. There was no association between NETs and PaO_2/FiO_2 ratio at the time of ICU arrival (R^2=0.01236, p=0.5379), days on the ventilator (R^2=0.03816, p=0.2760), or days in the ICU (R^2=0.1012, p=0.0712). There was no significant difference in NET levels between patients who developed PGD 3 and patients who did not develop PGD (p=0.1219).
a) NETs in recipient BW and days on ventilator

b) NETs in recipient BW and days in ICU

c) NETs in recipient BW and P/F ratio at ICU arrival
**Discussion**

Studying NETs in clinical bronchial wash samples taken post-reperfusion aimed to assess the clinical relevance of the findings from large animal models of lung transplantation; notably, whether NETs in the lungs associate with degree of lung injury at this time point. This study measured NETs in bronchial wash samples from recipients within soon after reperfusion. NET quantity was analyzed with regard to donor characteristics (donor type, gender, and use of EVLP in donor lung) as well as recipient outcomes (PaO\textsubscript{2}/FiO\textsubscript{2} ratio, PGD development, days on the ventilator, days in the ICU). No significant associations were observed in neither the donor characteristics nor the recipient outcomes.

A strength of this study was that it is representative of the lung transplant recipient population. 20% of the recipient samples were from DCD recipients, which is slightly higher than the reported 15% of DCD transplants performed in Toronto between 2012 and 2014 (Cypel, Levvey et al. 2015). The majority of recipient diagnoses were pulmonary fibrosis, CF, and COPD, as is seen in the general lung transplant population (Yusen, Edwards et al. 2016). Therefore, this study
population provides a meaningful snapshot of what is seen in clinical lung transplantation, and can help to characterize the role of NETs in this setting.

Contrary to the proposition that recipients of lungs which underwent EVLP would have less NETs in bronchial wash samples after reperfusion, there was no significant difference seen between EVLP and non-EVLP recipients. This finding implies that NETs may not be cleared from the lung during clinical EVLP, and therefore contribute to propagating NETosis in the recipient. DBD lungs are assessed on EVLP if they are deemed too injured for immediate transplantation; therefore, it is also plausible that NETs are removed from the lung during EVLP but this removal returns levels of NETs in the lungs to “baseline”, which is why there is no difference in NETs between lungs which did and did not undergo EVLP in the recipient bronchial wash after reperfusion.

There was no difference in NETs in bronchial wash samples between recipients of DBD or DCD lungs. This mirrors what was found in the EVLP perfusate study (i.e. no difference in quantity of NETs in DBD or DCD perfusate), and suggests that the donor type is not a factor in the quantity of NETs produced in the lungs.

NETs in bronchial wash samples analyzed in this study were not associated with recipient outcomes such as days on the ventilator or length of ICU stay, nor were they different between patients who developed PGD and those that did not. This finding contradicts the initial prediction.

The prediction that patients who developed PGD would have greater levels of NETs in the bronchial wash sample was supported by a report which describes NETs in BAL samples taken within 24 hours post-transplant as higher in PGD patients (Sayah, Mallavia et al. 2015). However, the study design presented in this report compared BAL samples from patients who developed “severe persistent PGD”, meaning PGD 3 at all time points within the first 72 hours post-transplant, matched to healthy controls for comparison. The study presented in this thesis did not match subjects, but rather was cross-sectional in nature. Furthermore, PGD 3 was defined as PGD 3 at 72 hours, not throughout the full 72 hours. The minority of patients in the study cohort presented with PGD 3 at 72 hours (n=3); of those, only one patient had PGD 3 at all time points. Therefore, it is possible that in a larger study cohort, or in a study specifically comparing severe PGD to no PGD as in the study by Sayah et al., NETs in bronchial wash samples would be associated with PGD. NETs were not associated with recipient length of ICU stay or days on the
ventilator, as was hypothesized; however, similar to PGD, this could be because the majority of the study cohort demonstrated fairly good outcomes post-transplant.

PaO$_2$/FiO$_2$ ratio at the time of ICU arrival was chosen for analysis because this time is very shortly after the bronchial wash was performed in the patients. Therefore, this analysis was assessing the relationship between NETs in the bronchial wash and oxygenation. No association was found, suggesting that while NETs may be associated with other aspects of acute lung injury, they do not directly contribute to hypoxemia.

This study is limited by the lack of data ascertaining cause-and-effect of NETs in the lungs: while the literature suggests that NETs are pathogenic in lung injury, this study can only associate different donor demographics and recipient outcomes with NETs. Furthermore, the study is inherently limited by the study cohort. As discussed previously, the standard of care in Toronto is to assess all DCD lungs by EVLP. For this reason, there is no non-EVLP DCD group for comparison. Therefore, while this study presents an accurate portrayal of clinical lung transplantation, it cannot fully characterize the role of NETs from a scientific standpoint. Finally, this study may be limited by the sample collection process. The bronchial wash procedure is not completely standardized, and thus may be subjective to the surgeon performing the bronchial wash. This could affect the quantity of NETs detected in the bronchial wash samples.
3.4 Conclusions

These studies examine NETs in clinical perfusate and bronchial wash samples. Examination of clinical samples presents both a challenge and an opportunity: there are numerous unknown environmental, genetic, behavioral and biological factors that could influence the detection of a single protein or cell—or, in this case, NETs—in a given individual. However, by the same token, the results are much more representative of the general population than an animal model may be.

Importantly, these studies showed that NETs can be detected in EVLP perfusate and are associated with recipient days on the ventilator. This is a twofold novel finding, as it is both the first time NETs have been measured in clinical EVLP perfusate as well as the first time donor lung NETs have been assessed as a biomarker for lung transplant outcomes.

NETs in recipient bronchial wash samples were not associated with the donor characteristics and recipient outcomes analyzed in this study; however, this is a setting of multifactorial injury and thus this result does not necessarily mean that NETs are not useful biomarkers in recipients as well as in donor lungs, but suggests that NETs may be reflective of just one part of the injurious processes occurring in the graft post-reperfusion.
Chapter 4: General Conclusions
The body of work presented in this thesis aimed to elucidate the role of NETosis in lung transplantation, in large animal pre-clinical models as well as clinical studies. Overall, this thesis aimed to address the hypotheses that NETs can be produced in donor lungs before transplantation as well as after reperfusion in the recipient, that NETs in donor lungs reflect the degree of donor lung injury, and that NETs can be measured in EVLP perfusate and used to predict recipient outcomes. The analysis of NETs in various swine models characterized NETs in the lungs at various stages of transplantation: in the donor lungs and in EVLP perfusate, in recipients after transplant, and in a model of transplant-related ECMO and aspiration injury. The study of NETs in EVLP confirmed the prediction that NETs can be detected in donor lungs and reflect donor lung injury, and that NETs in EVLP perfusate reflect the severity of lung injury. This finding was crucial to developing the study of NETs in clinical EVLP perfusate.

Surprisingly, alpha-1 antitrypsin treatment did not attenuate NETosis in a 72-hour survival ischemia-reperfusion injury model. In fact, alpha-1 antitrypsin seemed to encourage NET production, or suppress NET clearance, in a 4-hour survival model of ischemia-reperfusion injury. This finding contrasted the initial idea that the anti-inflammatory effects of alpha-1 antitrypsin may act on NETosis.

Another unexpected but informative result from the swine lung transplant studies was the discovery that neutrophils isolated from pigs in these models did not respond to the same NETosis stimuli commonly used for NETosis studies in human neutrophils. Interestingly, the same animals produced NE-DNA complexes, used as a marker for NETs, which were captured by NE-DNA ELISA in all the models included in this study. The lack of response of isolated neutrophils suggests that swine neutrophils undergo NETosis in a different manner than human neutrophils, but still respond to injurious stimuli in vivo. This prompted the development of two clinical studies to further assess the role of NETosis in lung transplantation.

NETs were detected in 200 clinical EVLP perfusate samples. This is the first description of NETs in clinical EVLP perfusate. Perfusate levels of NETs correlated with recipient days on the ventilator and length of ICU stay. This finding confirmed that NETs may be able to predict recipient outcomes. Interestingly, upon closer analysis of the study cohort, predictive patterns were seen in the DCD and female groups, but not in the DBD or male groups. Therefore, NETs may be produced in donor lungs differently based on the mechanism of donor death, or due to sex hormones present in the donor. This study represents the first examination of NETs in clinical
EVLP perfusate, and the first study of the predictive capacity of NETs in lung transplantation. Allocation of donor lungs aims to be as precise and efficient as possible; as such, the ability to predict recipient outcomes based on donor characteristics and biomarkers available in the perfusate carries highly relevant implications for improving donor lung allocation strategy and management.

Interestingly, the association of NETs in EVLP perfusate and recipient outcomes varied between donor types: DCD lungs were associated with recipient days on the ventilator and length of ICU stay, while DBD lungs were not. This finding may reflect the mechanisms of lung injury sustained during the death process, which varies biologically between brain death and cardiac death. Therefore, NETs may be useful as a biological representation of the type of lung injury sustained by donor lungs. However, in terms of predictive ability, NETs may be more useful as a predictor of recipient outcomes specifically in DCD lungs.

NETs are present in recipient lungs shortly after transplantation, as shown through detection of NETs in recipient bronchial wash samples. However, NETs in this study cohort were not associated with recipient outcomes, suggesting that the association between NETs and recipient outcomes seen in EVLP perfusate samples does not carry through the transplantation process. This study demonstrated that NETs are not closely associated with hypoxemia as measured by PaO₂/FiO₂ ratio; however, association of NETs with other recipient outcomes (such as development of PGD) was not apparent, and this analysis was perhaps limited by the study design.
Chapter 5: Future Directions
One important question that remains unanswered from this body of work is the why such a pronounced difference was observed in healthy pig and healthy human neutrophil response to NETosis agonists. The species difference is one plausible theory, but the exact difference in mechanisms remains unanswered. This is a relevant discussion to have in the field of lung transplant research, as swine are commonly used as large animal models for pre-clinical studies. Understanding where and how we are different from these animals is important for translating pre-clinical research to the bedside. In this specific scenario regarding NETosis, further investigations could be done comparing the response of isolated swine and human neutrophils to a wider range of agonists, including bacteria. As different NETosis agonists trigger different pathways (i.e. NADPH oxidase-dependent or -independent), comparing the response of a wide variety of agonists would shed light on which pathways are activated.

Understanding why only certain neutrophils undergo NETosis will be crucial for future studies. A first step is to determine characteristics of this subpopulation of neutrophils. Neutrophil subpopulation research is a rapidly evolving field (Fridlender, Sun et al. 2009); elucidating how these cells operate and react will offer insight into how NETosis is triggered.

Another question is the effect of alpha-1 antitrypsin on NETosis. The in vivo results of alpha-1 antitrypsin treatment were surprising, as similar or higher quantities of NETs were observed in treated pigs and in human lung EVLP treated with alpha-1 antitrypsin, which is contrary to our initial idea that alpha-1 antitrypsin would act to suppress NETosis. This could also be further elucidated in vitro, by incubating human neutrophils with alpha-1 antitrypsin and assessing the response to agonists. Again, comparing a wide variety of agonists would assist in determining the precise pathways in which alpha-1 antitrypsin acts with regard to NETosis.

This thesis presents novel knowledge on how EVLP can influence NETs in donor lungs, and how NETs are associated with lung transplant recipient outcomes. However, this body of work does not address in detail the mechanisms of how this happens. Future studies should be designed to answer in greater depth how EVLP is affecting the neutrophils and NETs in the donor lungs. For instance, a report recently published by Zheng et al. demonstrates that donor non-classical monocytes are the mediators of neutrophil recruitment and subsequent PGD, through production and signaling of CXCL2 (Zheng, Chiu et al. 2017). This study presents a possible mechanism by which neutrophils may be triggered to undergo NETosis, and thus a possible target for therapeutics.
that could be administered during EVLP and prevent NETosis in the recipient. A study addressing this would be feasible using EVLP as a research platform.

In order to fully address the role of NETs in lung transplant recipient short-term outcomes, more investigation is required. Specifically, a larger study cohort may be useful for understanding the injurious effects of NETs in recipients. Another area of interest that would help to further elucidate the effect of EVLP on NETs in lung transplant recipients would be quantitate donor-derived and recipient-derived NETs in recipient lungs. A study differentiating between NETs from donors and recipients would be able to assess the NETs produced by donor lungs and transplanted into recipients against the NETs produced in recipients from ischemia-reperfusion injury, and thus inform possible future therapeutic intervention.

Another area not fully addressed by this thesis is the possible benefits of NETs in lung transplant recipients. Although the evidence strongly points towards NETs being harmful in this context (Sayah, Mallavia et al. 2015), it is important to remember that NETs hold beneficial roles with regard to pathogenic invasion. This thesis study did not address infection in recipients, as it focused instead on the effects of ischemia-reperfusion injury in lung transplant recipients; however, examining the possibility that NETs could be combatting infection in this immunosuppressed patient population is important to consider before designing therapeutics targeted towards NETs.

Finally, this thesis suggests that NETs may be useful as a predictor of lung transplant recipient outcomes. However, a more rapid quantitative test for NETs in EVLP perfusate would be needed if this concept were to be applied clinically. The NE-DNA ELISA used throughout this thesis is limited in that it takes more time than would be ideal to make a decision regarding EVLP outcome. With the growing knowledge of NETs in clinical pathologies, developing a rapid, reliable, quantitative method for measuring NETs in biological samples will be essential for translating so-called “lab bench” knowledge to the patients’ bedside.
References


Appendix I: Contributions

Dr. Huiqing Lin, Dr. Yui Watanabe, Dr. Mamoru Takahashi and Andrew Cheung led the surgical studies assessing lung injury in swine EVLP. Dr. Ilker Iskender led the study assessing alpha-1 antitrypsin in lung transplantation with 4-hour survival. Dr. Andrea Mariscal led the study assessing alpha-1 antitrypsin in lung transplantation with 72-hour survival. Dr. Robert Qaqish and Dr. Lorenzo Del Sorbo led the aspiration injury and ECMO study. Dr. Meraj Khan assisted with study design, analysis, and conductance of in vitro NETosis assay experiments. Guan Zehong and Xiaohui Bai provided feedback for NE-DNA ELISA development. Ricardo Zamel assisted with clinical database extraction.

Drs. Tereza Martinu, Stephen Juvet, Marcelo Cypel and Jussi Tikkanen provided feedback, guidance, and suggestions throughout the project. Dr. Andrew Sage contributed to the human studies design, assisted with data collection, and contributed to study design and analysis. Dr. Andrea Mariscal assisted with study design, sample collection, performing the assays, analyzing the data, and reviewing the thesis. Drs. Mingyao Liu and Nades Palaniyar were PAC committee members and provided invaluable feedback and guidance throughout the project, especially with regard to NETosis and lung transplant biology, respectively. Dr. Shaf Keshavjee supervised, supported, and provided feedback for the thesis throughout this project.

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