Alpha 1 Antitrypsin Treatment of Donor Lungs:
A Translational Pathway to Clinical Application

by

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Abstract

Lung transplantation is a life-saving therapy for patients suffering from end stage lung diseases. Despite the many advances in the field, there is a clear shortage of donors primarily explained by the low utilization rate of the donor lungs offered for transplant. This underutilization is mainly a consequence of the surgeons being cautious about using more marginal lungs, because of the concern that patients will develop primary graft dysfunction (PGD) after transplant. PGD accounts for 30% of the mortality seen during the first month after transplant and it is also related to worse long term survival. Ischemia reperfusion injury (IRI) is a well-known contributing factor to the development of PGD. There is currently no therapy to prevent or treat PGD. In order to examine potential therapies to decrease IRI and reduce the incidence of PGD, there is a need for a relevant large animal lung transplant survival model.

A1AT is a serine protease inhibitor, its main function is to act as an antiprotease for neutrophil elastase. It acts like an acute phase protein increasing its concentration during inflammation. A1AT as a drug was purified from human plasma for A1AT deficiency patients. Subsequent studies have shown that A1AT has anti-inflammatory effects in different settings. We have been studying the
effect of A1AT in ischemia reperfusion injury in a systematic translational research approach testing its effects in progressively more complex models. Our hypothesis was that A1AT administered before transplantation in an attempt to reduce ischemia reperfusion injury would not have any detrimental side effects, and that A1AT dispensed to EVLP perfusate would improve human donor lungs that were previously rejected for transplantation.

The first part of the thesis is centered in developing a relevant pig lung transplant survival model to later on be able to test potential therapeutics to prevent PGD.

The second part of this work details the studies done to test alpha-1 antitrypsin (A1AT) in the pig single lung transplant survival model. Using the previously developed pig lung transplant survival model we were able to prove that A1AT is safe, well tolerated and if given to lung transplant recipient animals prior reperfusion, has beneficial effects including faster recovery and improved lung function after transplant.

For the third part of this thesis we used ex vivo lung perfusion (EVLP) as a platform to treat human lungs with A1AT. We explored the effect of A1AT treatment given during EVLP to injured human lungs that were not suitable for transplantation. For each case we divided the double lung block and treated one of the lungs in a randomized and blinded fashion. Using this approach we were able to assess the treatment effect of A1AT in the treated lung compared with the untreated one (both from the same donor), as well as compare all cases together (treatment vs control). A1AT was shown to have a beneficial effect on lung function measured by oxygenation function (pO2) and compliance. Biologically, A1AT treatment reduced lung endothelial injury (protected tight junctions) and decreased important pro-inflammatory cytokines that are relevant to endothelial dysfunction.
A1AT has been proven to have beneficial anti-inflammatory and immunomodulatory effects. A1AT is an already approved drug that has shown to be well tolerated and has the potential to improve human donor lungs that were previously rejected for transplantation.

Based on our findings, we are confident to move forward with a randomized prospective clinical trial to use A1AT to prevent PGD in human lung transplantation.
Acknowledgments

Foremost, I would like to express my sincere gratitude to my supervisor Dr. Shaf Keshavjee for his guidance and support in addition to providing me with the opportunity to lead this exciting translational research. Most importantly I want to thank him for introducing me to this amazing world of cutting-edge research that made me realize that lung transplant is a lot more than an exciting surgery. He has been and continues to be the best role model as a surgeon-scientist.

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This project would not be a reality without the help of my colleagues in the lab: Allen Duong thank you for hard work with all the sample collection and processing, Marcos Galasso and Aadil Ali thank you for your help setting up running the ex vivo lung perfusion experiments. Thanks to Andrew Sage, William Klement and Ricardo Zamel for his advice, to Manyin Chen for his support during the pig surgeries and Soltanieh, Sahar for the endothelial cell cultures. I also thank Antti Nykanen for his help and support during this last year. During this years I have been lucky enough to meet many students, fellows and technicians from very different backgrounds that have made this an easier and enjoyable journey. I would specially like to thank you Lindsay Caldarone, my research partner, thank you for all your help and support during the experiments that seem to be endless, there is no way I could have done any of them without you being there, but specially thank you for your friendship that I am sure will continue over the years.

Last but definitely not least, I thank my family my mother, father, my brothers and specially my sister and life friend for their great moral and emotional support. My husband who proof read this thesis and helped arranging the format, tables and figures.
Dedication

This thesis is dedicated to my husband Iñaki. He always encouraged me to pursue my dreams and has been holding my hand all the way even if that meant moving countries or bouncing our baby while working so I could be in the operating room. This thesis is also dedicated to my three kids, the center of my life, Iker my big intelligent boy, my sweet and strong Sofia and my Canadian baby Anna who was born during the preparation of this thesis and whose smile always makes me happy. This work is for you my incredible family.
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<table>
<thead>
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<tbody>
<tr>
<td>A1AT</td>
<td>Alpha 1 antitrypsin</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>DCD</td>
<td>Donation after cardiac death</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EVLP</td>
<td>Ex vivo lung perfusion</td>
</tr>
<tr>
<td>ET1</td>
<td>Endothelin 1</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>CCL2</td>
<td>Chemokine C-C motif ligand 2</td>
</tr>
<tr>
<td>HLtx</td>
<td>Heart Lung Transplant</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischemia reperfusion injury</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NE</td>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PGD</td>
<td>Primary graft dysfunction</td>
</tr>
<tr>
<td>SERPINA</td>
<td>Serine protease inhibitor</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens protein-1</td>
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Chapter 1. Introduction
In this chapter we will review the history of organ transplantation, indications and contraindications of lung transplantation, donor selection criteria and strategies to increase the available donors. We will also analyze the lung organ preservation strategies, primary graft dysfunction and ischemia reperfusion injury. The last part of the chapter will review about Alpha 1 Antitrypsin and its effects.

1.1. Lung Transplantation

Over the last decades organ transplantation has become a reality, giving an opportunity to patients suffering from end stage organ failure to extend life and improve quality of life. Lung transplantation has overcome the initial major limitations that where preventing it from being a real life saving therapy.

Since the first long-term successfully lung transplant, performed in Toronto\(^{(Reichart et al. 1987)}\) we have witnessed enormous advances in surgical technic, organ preservation and immunosuppression that have led to an increasing number of lung transplants performed each year. Between 1998 and 2000 the number of lung transplants performed each year was around 1900. According to the last registry, in 2016 more than 4500 lung transplants were performed worldwide\(^{(Chambers et al. 2018)}\). The Canadian Institute for Health Informatics in his annual statement reported that between January 1st 2017 and December 31 2017, 341 lung transplants were performed in Canada in comparison to 247 done during the same period in 2013.\(^{(Informatics 2017)}\)

The number of patients waiting for a transplant however, continues to increase in greater proportion than the number of available donor lungs, which translates to more patients dying on wait list.\(^{(Informatics 2017)}\)
1.1.1. Organ Transplantation History

During the 18 and 19\textsuperscript{th} century researchers experimented with organ transplantation. Following many failures, successful solid organ transplant was achieved after the discovery of the fundamentals of acquire immune tolerance and graft rejection by Peter Brian Medawar in the 1940s\cite{Ribatti2015, Brent2016}. Bone, skin and corneas were the first successful transplants achieved between 1900 and 1920. The first successful human kidney transplant was achieved by Dr. Joseph E. Murray in 1954 in Boston, he accomplished it using identical twins as donor and recipient to avoid the immune response all together\cite{Merrill1984}. The first positive studies in heart transplantation were done by Dr. Norman Shumway in Palo Alto, California, he developed a surgical technic in which he cooled the heart and left part of the atrium \textit{in situ} to reduce the number of anastomoses required during the transplant, using animals as experimental models\cite{Dong1965}. However the first human heart transplant was performed in Cape Town by Christian Bernard, using Dr. Shumway’s technic.\cite{Barnard1968}

The Russian scientist Vladimir Demikhov in 1946 performed the first heart and one pulmonary lobe transplantation. He then transplanted the heart and both lungs, the heart alone and a lung alone. These experiments were performed without cardiopulmonary bypass in dogs. The mortality was high but some of the dogs were able to survive for 4-6 days. In 1947 Demikhov performed the first isolated lung transplantation, the bronchial anastomoses was performed by hand suture first and then with a mechanical suturing device. Demikhov believed that preservation of the nerves diaphragm was vital and he was finally able to maintain the lung transplanted dogs alive for up to one month. Demikhov thought preservation of the bronchial arteries and nerves was not necessary.\cite{Konstantinov1998, Matskeplishvili2017} In 1950 Metras in France developed a lung transplant technic in dogs that included harvesting and anastomosis the left bronchial artery to provided blood
supply to the bronchial anastomoses, we also described the left atrial anastomoses instead of single pulmonary vein sutures. (Cooper 1990) Hardy published in 1963 further experiments of lung autotransplantation and homotransplantation in dogs investigating the role of bronchial arteries, lymphatics and vagus nerves, as well as the use of methotrexate and azathioprine for immunosuppression. He concluded that lung homotransplantation was clinically feasible, but in acute or self-limiting conditions. (Buecherl, Nasseri, and Von 1964) In 1963, Hardy was able to accomplish the first human lung transplantation at the University of Mississippi Medical Center. The donor he used had a cardiac arrest and the lung was retrieved post mortem. The recipient was an emphysema patient that also had a squamous cell carcinoma in the left lung and kidney disease, the immunosuppression consisted on azathioprine, prednisone and radiation (cobalt therapy) to the mediastinum he was able to survive for 18 days and died because of renal failure. (Aru et al. 2004; Hardy 1999) During the following 15 years several attempts of transplanting lungs were made without achieving long-term survival. Nine lung transplant recipients lived for more than two weeks and one patient was discharged from the hospital in 1968. The majority developed bronchial complications (bronchial anastomosis dehiscence) that compromised their lives. Derom, in Belgium in 1968 was able to perform a left single lung transplant, the 23 year old man with silicosis was able to survive for 10 months, he died of bronchopneumonia without showing any signs of acute rejection, although there were some signs regarding chronic rejection. (Derom et al. 1971) The next step towards clinical lung transplantation was done by Joe Cooper in Toronto in 1978, he performed a right single transplant in a man that suffered severe burns, the patient was placed on extracorporeal membrane oxygenation before transplant and the support was maintained 4 days after the transplant procedure. Two weeks after transplant the patient was extubated and was able to be ambulatory, but died during the third week due to a bronchial anastomosis dehiscence. (Nelems
After analyzing all the lung transplant trials that survived longer than one week, the Toronto group realized that cause of death was mainly related to complication of the airway, and decided to further investigate the possible causes of bronchial dehiscence. Among the possible causes of inappropriate bronchial healing, they determined that limited bronchial blood supply due to transection of the bronchial arteries during procurement causing ischemia of the anastomosis could be one of the most important reasons. Other factors that could be affecting the correct anastomosis healing could also be rejection and the effect of the immunosuppressant drugs. Animal studies performed by the Toronto group revealed the negative effect of azathioprine given as immunosuppressant after transplantation. On the other hand, cyclosporine did not compromise the healing of the bronchial anastomosis. In order to avoid distal stenosis of the bronchus secondary to ischemia of the airway, they put omentum around the bronchial anastomosis in an attempt to provide collateral circulation to the bronchial anastomosis. After the introduction of cyclosporine, the first in block heart-lung transplant (HLtx) long term survival was performed by Bruce Reitz and his team at Stanford University. The patient had pulmonary hypertension, although she developed some episodes of rejection, lung function remained stable. On November 7th 1983, the Toronto group led by Cooper, performed the first successful single lung transplant (SLtx) on a 58-year-old male with Pulmonary Fibrosis. They used their previously published technic were the omentum was transposed into the chest in able to cover the bronchia anastomosis. The patient was discharged six weeks after transplant and went back to an active life, he died because of renal failure 6.5 years later. Following various successful SLtx the Toronto Group decided to go ahead and do a double lung transplant using the en bloc technic developed in their laboratories. After publishing the initial
experience of 7 patients that received a double lung transplant using this technic, (Cooper et al. 1989) they realized that the patients were developing airway complications despite covering the anastomoses with omentum, that’s when the bilateral sequential transplantation approach was initially used. Using this new technic they were able to reduce the airway complications, (Pasque et al. 1990)

1.1.2. Indications and Contraindications for Lung Transplantation

Lung transplantation is now a days accepted as a treatment option for patients with severe end-stage respiratory diseases without other medical or surgical therapy options. The most common indications include: chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, cystic fibrosis, alpha-1 antitrypsin deficiency, idiopathic pulmonary arterial hypertension as well as re-transplantation.

As a general rule lung transplantation is meant to improve not only survival but also quality of life for those patients. The International Society for Lung and Heart Transplantation consensus for lung transplant indications was published in 1996 and 2006, (Orens et al. 2006) more recently the ISHLT established a guidelines update in 2014 for lung transplant recipient selection, where the candidates should be considered for lung transplantation if the established criteria are met.

Ideally, lung transplantation will improve both the survival and quality-of-life (QoL) of a patient with end-stage lung disease. To achieve this, the patient should have severe end-stage lung disease despite optimal therapy and not have any other disease or major organ dysfunction that will limit survival unless a combined organ transplant is considered. The time to place a patient on wait list is crucial, since the transplant surgery implies risks that that shouldn’t be taken when the patient has other treatment options. Similarly, putting a very sick patient on the wait list too late may lower the possibilities of it being successful. The 2014 ISHLT consensus statement for recipient
selection (Weill et al. 2015) suggests lung transplant consideration for adult candidates when the all of the following general criteria are met:

1. High (>50%) risk of death from lung disease within 2 years if lung transplantation is not performed.
2. High (>80%) likelihood of surviving at least 90 days after lung transplantation.
3. High (>80%) likelihood of 5-year post-transplant survival from a general medical perspective provided that there is adequate graft function.

According to the same ISHLT 2014 consensus there are some absolute and some relative contraindications for lung transplantation.

Absolute contraindications are:

1. Recent history of malignancy. Patients should have a 2-year disease-free interval in addition to low predicted risk of recurrence after lung transplantation, or a 5-year disease-free interval for patients with a history of hematologic malignancy, sarcoma, melanoma, or breast, bladder, or kidney cancer.
2. Untreatable significant dysfunction of another major organ system (heart, liver, kidney, or brain) unless a combined organ transplant can be performed.
3. Uncorrected atherosclerotic disease with end-organ ischemia or dysfunction and/or coronary artery disease not susceptible to revascularization.
4. Acute medical instability, including, but not limited to, acute sepsis, myocardial infarction, and liver failure.
5. Bleeding diathesis that can be remedied.
6. Chronic infection with highly virulent and/or resistant microbes that are badly or not controlled pre-transplant.
7. Active Mycobacterium tuberculosis infection.
8. Significant chest wall or spinal deformities that cause severe respiratory restriction
9. Class II or III obesity
10. Present non-compliance of medical treatment or a history of recurrent or sustained episodes of non-adherence to medical therapy.
11. Psychiatric and or psychologic conditions that are associated with the incapacity to cooperate with the medical health care team and/or follow medical therapy.
12. Lack of reliable social support system.
13. Very limited functional status with poor rehabilitation potential
14. Substance abuse or dependence (including alcohol, tobacco, marijuana, or other illicit drugs).

Relative contraindications include:

1. Age >65 years in addition to physiologic reserve and/or other relative contraindications. Although there is not strict upper age limit, patients over 75 years old are not commonly candidates in most cases.
2. Class I obesity especially if it is central obesity.
3. Progressive or severe malnutrition.
4. Severe, symptomatic osteoporosis.
5. Extensive former chest surgery with lung resection.
6. Mechanical ventilation and/or extracorporeal life support (ECLS). Though, selected candidates without other acute or chronic organ dysfunction can be successfully transplanted.
7. Colonization or infection with highly resistant or highly virulent bacteria, fungi, and some strains of mycobacteria.
8. Hepatitis B and/or C infected patients can receive a lung transplant if there isn’t any significant clinical, radiologic, or biochemical signs of cirrhosis or portal hypertension and who are stable on appropriate treatment.

9. Human immunodeficiency virus (HIV) positive patients, can be considered for lung transplantation if the disease is under control with undetectable HIV- RNA, and the patients are compliant on combined anti-retroviral therapy.

10. Infection with Burkholderia cenocepacia, Burkholderia gladioli, and multi-drug–resistant Mycobacterium abscessus if the infection is appropriately treated before surgery and there is a reasonable expectation for adequate control postoperatively.

1.1.3. Lung Transplantation Outcomes

According to the latest ISHLT registry, since 1985, and until 2017, more than 65000 lung transplants were performed. The number of transplants by year and procedure type is shown in Figure 1.

Figure 1 Adult and pediatric lung transplants

Number of transplants by year and procedure type. Adapted from Chambers et al JHLT. 2018 Oct; 37(10): 1155-1206 (Chambers et al. 2017)
According to the 2017 report of this registry, adults that received a lung transplant between January 1990 and June 2015 had a median survival of 6 years. If we take into consideration just the patients that survived 1 year after transplant the conditional median survival was 8.1 years. Bilateral lung transplant recipients had better survival than unilateral recipients in the unadjusted analyses starting in the first year after the surgery. Bilateral and unilateral lung transplant recipients had 93% to 94% survival at 1 month, at 3 months for the double lung transplant recipients survival was 90% and for the single lung transplant recipient 88%; 1 year after transplant, the group that received 2 lungs had a 82% survival and the group that received just one lung 78% the difference in survival between the 2 groups increases during the following 14 years being 59% and 48% at 5 years, and 41% and 23% at 10 years respectively (p= 0.0001, Kaplan-Meier curve). Patients that belonged to the retransplant group had an inferior survival if compared with the ones that received a lung transplant for the first time, the retransplant survival at 1 month was 86%, at 1 year 67%, 40% at 5 years, and 21% at 10 years. (figure 2)
The main causes of death after lung transplantation change depending on the time after transplantation. The leading causes of death during the first 30 days are related to primary graft dysfunction (PGD) (26.9%), and technical issues during the surgery itself (23.3%), the third most frequent cause of death during that period of time is infection (1.9%). During the first year, infection accounts as the first cause of dead graft failure remains the second and multi organ failure the third cause of dead. 5 years after transplant the main causes of dead are infections (21.7%), bronchiolitis obliterans syndrome (BOS) (20.6%), graft failure and malignancy (10.1%).

1.2. Lung Donors

1.2.1. Donor Selection Criteria

The traditional donor criteria that are currently in use were established in the 1980s during the early years of the lung transplantation era. Today there are many centers that still use dose criteria to judge if a lung donor can be used for transplantation. These criteria include age below 55 years old, an arterial partial pressure of oxygen (PaO₂)/FiO₂ ratio greater than 350, no or minimal smoking history, a clear chest X-ray, clean bronchoscopy, gram stain negative, less than 5 days of intubation and a minimal ischemic time. (Reyes et al. 2010; Orens et al. 2003; Filosso et al. 2006) See Table 1

Nowadays most centers agree that the initially established criteria are too rigorous and are in favor of using donors that do not completely meet the traditional empiric criteria called extended criteria donors (ECD). (Chaney et al. 2014; Botha 2009) Although the results of using ECD have shown diverse outcomes, the use of this type of donors has become acceptable especially if the risk of dying on wait list is high. (Botha et al. 2006; Ehrsam et al. 2017; Bhorade et al. 2000; Meers et al. 2010; Pierre et al. 2002; Lardinois et al. 2005)

The use of ECD has effectively increased the donor pool with acceptable post-transplant outcomes. (Chaney et al. 2014; Cypel and Keshavjee 2013)
### Table 1 Traditional Donor Lung Criteria

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<tr>
<td>Age &lt; 55</td>
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<tr>
<td>ABO blood type compatibility</td>
</tr>
<tr>
<td>Smoking history &lt; 20 years</td>
</tr>
<tr>
<td>Clear chest x-ray</td>
</tr>
<tr>
<td>((\text{PaO}_2)/\text{FiO}_2) ratio &gt; 300 at \text{FiO}_2 100 and PEEP 5</td>
</tr>
<tr>
<td>Absence of chest trauma</td>
</tr>
<tr>
<td>No evidence of aspiration or sepsis</td>
</tr>
<tr>
<td>No previous cardiothoracic surgery</td>
</tr>
<tr>
<td>No organism on donor gram stain</td>
</tr>
<tr>
<td>No purulent secretions in bronchoscopy</td>
</tr>
<tr>
<td>Ischemic time &lt; 4h</td>
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</tbody>
</table>


After the donor lungs are considered for transplantation, depending on the allocating system, the lungs are offered to an institution. A blood test is the first that its performed to determine the blood type and screen for possible infectious diseases. Some institutions do an HLA (human leukocyte antigens) blood test to determine tissue type and further evaluate compatibility. A chest x-ray is taken to assess for pleural or parenchymal abnormalities, in some cases a chest computed tomography (CT) is done to further rule out parenchymal or anatomical anomalies. A bronchoscopy is performed to assess for signs of infection, aspiration or anatomical abnormalities, a donor bronchial wash (BW) or bronchioalveolar lavage (BAL) in collected to be further analyzed. During the retrieval surgery, the surgeon palpates the lung to exclude macroscopic...
consolidations, infiltrates, or nodules and assesses it for compliance and edema by evaluating lung deflation.

1.2.2. Donor Shortage and Strategies to Increase Available Donors

Despite the increasing number of donors, there is still a meaningful discrepancy between the demand and availability of donor lungs. Success in lung transplantation at improving survival and enhancing quality of life has translated in proliferation of transplant programs and extension of the indications, increasing the number of patients being listed as potential lung recipients. As a result, wait list mortality has increased. Wait list mortality differs between countries and centers and is reported to be above 16% for some diagnoses. One determinant factor in the organ shortage is the relatively low utilization of lungs, retrieval rates reported for the “Eurotransplant” zone is around 28% and 20% for the USA and UK.\(^{(\text{Van Raemdonck et al. 2009; Gomez, Perez, and Manyalich 2014})}\)

To help overcome shortage of donors and the low utilization rate, different strategies have been developed.\(^{(\text{Knoll and Tinckam 2015})}\)

According to the Canadian Institute for Health Information, the deceased organ donation rate reported in 2016 was 20.9 donors per million population. Although this number shows an increase of 42% if compared with the data reported in 2007 (14.7 donors per million population), this rate remains low in comparison with other countries like Spain that reported 46.9 donors per million population.

Some of the strategies that could increase the number of available donor include: national initiatives, use of extended criteria donors, and the use of other donor sources like donors after cardiac dead (DCD).
Figure 3 Organ Donor Rates in Canada 2007 - 2016

The donor organ rate in Canada increased by 42% between 2007 and 2016 according to the Canadian Institute for Health Information. *Results from 2012 to 2016 are supplemented with data from Transplant Québec. Data from the Canadian Institute for Health Information

**Increasing Organ Donation Rates in Canada**

Canada has stipulated initiatives to boost organ donor rates including:

- A national registry containing information about donor and recipients that will help improve efficiency in identifying compatible recipients and greater number of donors.
- A national real-time wait list similar to the one implemented by the United Stated (UNOS). The list of potential recipients is ranked according to some objective medical criteria including blood and tissue type, size and medical urgency as well as patient specific situation and time spent on waiting list.
- A national organ waitlist (NOW) was started in June 2012 as a real-time online listing for heart, lung, pancreas bowel, liver and multi organ). The transplant programs can access the database to identify patients in critical status across
Canada. NOW is able to provide information about wait times and organ availability.

- The highly sensitized patient program started in 2013 as an effort to provide a registry for kidney transplant candidates that are highly sensitized and not easy to match.

- A living donor registry. For bone marrow or circulating peripheral blood as stem cell sources.

- A national public umbilical cord bank as stem cell source was established in 2011.

- Establishment of presumed consent was been suggested as a way to increase the donor rates. In this model consent to donate is presumed unless a person has expressly indicated otherwise.

- A mandatory referral for all brain death (and possible cardiac death) patients as well as eminent deaths to a local organ procurement organization. This required reporting has been or is in the process of being implemented in all Canadian provinces except for Saskatchewan and Newfoundland.

- Professional specific training in donor recruitment, trained coordinators that identify potential donors and approach families for consent. Some provinces have implanted donation physician programs.

- A mandatory declaration that requires that individuals declare whether they want to consent to organ donation.

- A national public awareness campaign has been established to facilitate informed choice and to increase involvement in the donation and transplant process.

The donation rate in Canada has been low if compared with other countries. The coordination of donation and transplant in Canada is more challenging due to the large territory as well as the lack of legislated mandates on healthcare delivery. Instead of a national healthcare system there are 10
provincial and 3 territorial systems that are administered by 13 separate Ministries of Health.\textsuperscript{(Knoll and Tinckam 2015)} Some strategies have been proposed and other have already been implemented. The institution of trained coordinators and the mandatory referral of brain death have been translated in an increase in the donor rate in the recent years. However the total number of organ donors is still low if compared with higher performing countries and it doesn’t meet the needs. Further initiatives should be implemented in order to increase the total number of donors as well as the organ utilization rate.

**Extending the Donor Pool**

Several methods have been used in an attempt to expand the donor pool. Currently the minority of the lungs being offered meet the ideal criteria established in the 1980s. Donor lungs that don’t meet the strict criteria are extending criteria donors (ECD). According to the United Network for Organ Sharing (UNOS) analysis published by the Cleveland Clinic group, only 44\% of the donor lungs used in the United States meet the ideal criteria the other 56\% were ECD.\textsuperscript{(Reyes et al. 2010)} The most frequent criteria that are not always met are age, smoking history, abnormalities on chest x-ray and positive gram stain on bronchoalveolar lavage. Different centers have published the use of marginal donor with diverse results.\textsuperscript{(Pierre et al. 2002; Lardinois et al. 2005; Bhorade et al. 2000; Sundaresan et al. 1995; Gabbay et al. 1999; Moreno et al. 2014)} However the comparison in between the studies is difficult since the extension of the criteria are different depending on the center.\textsuperscript{(Snell et al. 2008)} Signs of infection in chest x-ray and or bronchoscopy (purulent secretions) have been correlated with worse post-transplant outcomes in several studies, particularly in addition to low donor pO\textsubscript{2} (<300) \textsuperscript{(Pierre et al. 2002; Lardinois et al. 2005)}. Regarding the smoking history, various studies have shown a relationship between donor history of cigarette smoking and worse early outcomes\textsuperscript{(Oto et al. 2004; Diamond et al. 2013)}, but there are several other investigators that have shown that donors with smoking history can be
used safely.\textsuperscript{(Shigemura et al. 2013; Taghavi et al. 2013; Berman et al. 2010)} Consistent outcomes have been shown using donors within the age range of 18-64 years. Retrospective reviews from the UNOS database from 2000-2010 showed an increase in 1 and 3-year mortality for patients who received lungs from donors older than 65 years, they also documented an increase in bronchiolitis obliterans syndrome (BOS).\textsuperscript{(Bittle et al. 2013)} The Organ Procurement and Transplantation Network in the United States (OPTN) analysis showed no increase in 1 year graft failure for donors between 18 and 64 years. Donors over 64 were associated with increase graft failure 1 year after transplant but didn’t show more incidence of primary graft dysfunction (PGD).\textsuperscript{(Baldwin et al. 2013)} Overall the use of extended-criteria lungs has generally become acceptable, especially in weighing the risk balance to death while waiting for lungs to become available.

Use of alternative donor sources

In the last ten years, an increase in multiorgan organs have been accomplished by using donors after cardiac death (DCD).\textsuperscript{(Kootstra, Kievit, and Nederstigt 2002; Steinbrook 2007)} Between 2006 and 2008 the increase in DCD was 24\%.\textsuperscript{(Cypel and Keshavjee 2013)} After some dog experiments, Egan showed that the lung could remain functional for some time after death probably as a result of the oxygen reserve that is maintained in the alveoli.\textsuperscript{(Egan et al. 1991; Ulicny et al. 1993)} This reintroduce the concept in 1991 of DCD donors previously explored by Hardy during the first human lung transplant.\textsuperscript{(Hardy 1999)} Following Egans initial experience Ulcini continued to work in the DCD dog model.\textsuperscript{(Ulicny et al. 1993)} Rega \textit{et al.} used a pig DCD model to show that topically cooled lungs had better function compared to lungs that were just ventilated.\textsuperscript{(Rega et al. 2003)} In 2002 Watanabe \textit{et al.} successfully transplanted a DCD donor lung in a dog that was topically cooled for 2 h using cold air to fill the hemithorax.\textsuperscript{(Watanabe \textit{et al. 2002})} Steen used a pig DCD model maintaining the chest open to be able to cool the lungs with a saline slush inside the pleural spaces,
later Steen’s group decided to cool the lungs inside the pleural spaces with a continuous infusion delivered through two intrapleural chest tubes inserted in between two costal spaces. (Steen et al. 2003)

Inci et al. in Switzerland investigated the role of topical cooling with Perfadex, demonstrating that the group that after 3 h of death resulted in improved graft function compared to saline group. Functional parameters were comparable between saline and Perfadex groups after 1 h of warm ischemia. (Inci et al. 2008) In 2016 Martens et al. showed that steroids given before warm ischemia time or during ex vivo lung perfusion is able to reduce injury in DCD. (Martens et al. 2016) The first clinical experience using DCD was reported by Love et al in 1995. (PN 1995)

In 1995 after the increasing experience in DCD, the First International Workshop on DCD was hosted in Maastricht, Netherlands and four different categories of DCD were characterize, contemplating different technical and medical features Categories I (dead on arrival) and II (unsuccessful resuscitation) are uncontrolled donors and Categories III (cardiac arrest after withdrawal of life support) and IV (cardiac arrest in brain-dead donors). See Table 2.

Table 2 Donation after cardiac death: Maastricht categories for DCD

<table>
<thead>
<tr>
<th>MAASTRICHT CATEGORY</th>
<th>DESCRIPTION</th>
<th>CONDITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Dead on arrival at hospital</td>
<td>Uncontrolled</td>
</tr>
<tr>
<td>II</td>
<td>Unsuccessful resuscitation</td>
<td>Uncontrolled</td>
</tr>
<tr>
<td>III</td>
<td>Anticipated circulatory arrest</td>
<td>Controlled</td>
</tr>
<tr>
<td>IV</td>
<td>Circulatory arrest in patient previously declared brain dead</td>
<td>Controlled</td>
</tr>
<tr>
<td>V</td>
<td>Circulatory arrest in hospital</td>
<td>Uncontrolled</td>
</tr>
</tbody>
</table>
In 2001, Steen published a successful lung transplantation using a donor that died in the hospital after a cardiac arrest (category II DCD)\(^{(1)}\). In 2004 the Madrid group reported 2 successful lung transplantations from uncontrolled DCDs (category I)\(^{(2)}\) and in 2007 the series was updated including patients 17 patients transplanted using uncontrolled DCD and showing similar mid-term results\(^{(3)}\). Centers from around the world started using category lungs obtained after controlled withdrawal from life support (Category III) reporting good outcomes\(^{(4)}\).

In 2011, the International Society for Heart and Lung Transplantation (ISHLT) started a DCD Registry in order to: establish and standardize definitions of the time intervals related to the DCD donation process; compare survival outcomes between DCD lung transplants and DBD transplants; and analyze donor, recipient and other characteristics in DCD transplant that could be associated with short and long term outcomes.

In 2013, during the 6\(^{th}\) International Conference on Organ Donation after Circulatory Death held in Paris, the European Working Group proposed to update the initial Maastricht classification according to the new developments\(^{(5)}\). (See table 3)

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{CATEGORY} & \textbf{DESCRIPTION} & \textbf{CONDITION} \\
\hline
I & Found dead & Sudden unexpected cardiac arrest without any attempt of resuscitation by a life-medical team; WIT to be considered according to National life-recommendations in place; reference to in- or out-of-hospital life setting \\
I & IA. Out of the hospital & \\
I & IB. In hospital & \\
Uncontrolled & & \\
\hline
\end{tabular}
\caption{New Classification of Maastricht Categories for DCD}
\end{table}

\(^{(1)}\) Steen et al. 2003
\(^{(2)}\) Nunez et al. 2004
\(^{(3)}\) de Antonio et al. 2007
\(^{(5)}\) Thuong et al. 2016
### Categories

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>DESCRIPTION</th>
<th>CONDITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>Witnessed cardiac arrest</td>
<td>Sudden unexpected irreversible cardiac arrest with unsuccessful resuscitation life-by-a-life-medical team; reference to in- or out-of-hospital life-setting</td>
</tr>
<tr>
<td>II Uncontrolled</td>
<td>II A. Out of the hospital</td>
<td></td>
</tr>
<tr>
<td>II Uncontrolled</td>
<td>II B. In hospital</td>
<td></td>
</tr>
<tr>
<td>III Controlled</td>
<td>Withdrawal of life-sustaining therapy</td>
<td>Planned withdrawal of life-sustaining therapy; expected cardiac arrest. (medically assisted cardiac arrest)</td>
</tr>
<tr>
<td>IV Uncontrolled</td>
<td>Cardiac arrest while life-brain dead</td>
<td>Sudden CA after brain death diagnosis during donor life-management but prior to planes organ recovery</td>
</tr>
<tr>
<td>IV Controlled</td>
<td></td>
<td></td>
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</tbody>
</table>


The International Society for Heart and Lung Transplantation Donation after Circulatory Death Registry in his report in 2015 showed that controlled DCD lung transplantation is safe even without the use of ex vivo lung perfusion, however the use of EVLP could increase the utilization of extended criteria DCD donors and can help towards better outcomes in DCD Categories I and II as well and some high risk Category III donors. (Cypel et al. 2015)

Although the initial series with limited patients showed conflicting results, recent studies with a larger numbers of patients have been reported with satisfactory outcomes including the multicenter study published by Cypel et al. (Levvey et al. 2012; Cypel, Sato, et al. 2009; De Oliveira et al. 2010; Love 2012; Inci et al. 2018)

Cypel published a review from 10 centers (300 DCD transplants), and showed a 97% 30-day and
89% 1-year survival. (Cypel et al. 2015) These results are comparable current to short and long-term outcomes from DBD lung transplants. The number of centers using DCD has grown over the last decade, but DCD lung still remain underused.

**Improving the donor management**

Prior to the retrieval phase the main goal is to maintain the donor stable in order to preserve as many organs as possible and reduce the incidence of primary graft dysfunction. Aggressive management of the donor and standardization of donor management has been shown to increase the lung quality by improving pre-retrieval oxygenation. The protocol goals should be set for: mean arterial pressure, pH, oxygenation, sodium, glucose, single vasopressor use, urine output, and central venous pressure can increase the rate successful organ donation, particularly in lung transplantation. (Franklin et al. 2010; Angel et al. 2006; Venkateswaran et al. 2008; Shemie et al. 2006)

The current recommendations on donor management and lung preservation published by Munshi et al (Munshi, Keshavjee, and Cypel 2013) include:

- Ventilation with low tidal volume (6-8 mL/Kg).
- Fraction of inspired oxygen of 50%.
- Positive end expiratory pressure of 5-10 cm H₂O.
- Euvolemia with a target central venous pressure of 6-8 mmHg.
- Hemodynamic support: Initial vasopressor used for hemodynamic instability should be vasopressin. Norepinephrine, epinephrine or phenylephrine are second line of treatment.
- Hormone therapy replacement: methylprednisolone, vasopressin and desmopressin for diabetes insipidus and thyroid replacement.
1.3. Lung Organ Preservation


During the retrieval procedure, the donor is ventilated with \( \text{FiO}_2 \) 50\%, PEEP 5 cm\( \text{H}_2\text{O} \) and \( V_T \) 8 ml/kg. Standard median sternotomy is performed to gain access. Pleural spaces are entered and examined. Recruitment of all atelectatic lung segments is performed in situ by gentle massage of both lungs at the same that that the anesthesiologist inflates them to a sustained pressure of up to 30 cm \( \text{H}_2\text{O} \). Palpation of both lungs and visual evaluation of deflation capacity (recruitment with 20 cm\( \text{H}_2\text{O} \) or 30 cm\( \text{H}_2\text{O} \) for 30 seconds followed by opening of the airway to room air) is performed. The results of this examination, together with the latest results of the arterial blood gas, chest x-ray, and bronchoscopy, are communicated to the lung recipient implant surgeon for final decision making. The anticipated cross-clamp time and estimated arrival time to the recipient hospital is informed for logistical planning. For evaluation of unilateral or lobar dysfunction, individual pulmonary vein gas sampling can be useful and can be key at rescuing one well-functioning lung for a single lung transplant when the donor systemic blood gases are below optimal due to one severely damaged lung.

After the pericardium is opened, the superior vena cava is dissected up to its bifurcation into the innominate veins and is encircled using an 0-silk tie, the ascending aorta is detached from pulmonary artery (PA) and encircled with an umbilical tape. A purse string suture of 4-0 polypropylene suture (Prolene; Ethicon, Inc, Somerville, NJ) is placed midway between PA valve and the bifurcation in the main pulmonary artery (to later secure the PA flush cannula).
The donor is heparinized 3-5 min before cannulation. The lung flush solution (low potassium dextran, Perfadex®) is hung 30 cm above the level of the heart. The cannula is inserted into the PA positioning the tip before the PA bifurcation and is and connected to the infusion line. A bolus of prostaglandin E1 (500 mcg in 10ml normal saline) is injected into the main pulmonary artery. Once the blood pressure drops indicating, the aorta is cross-clamped. The superior vena cava is ligated. The inferior vena cava is then vented by complete transection above the diaphragm. The left atrium is vented by transecting the tip of the left atrial appendage creating a drainage hole. The cardioplegia for cardiac preservation and the pulmonary preservation flush are then initiated by administering it into the pulmonary arteries through the high-flow perfusion cannula at 30 cm height gravity pressure. The flush solution emanating from the atrial appendage is allowed to drain into the pleural space to provide additional topical cooling of the lungs. The lungs remain on the ventilator throughout this phase. At the end of the antegrade flushing procedure (60 ml/kg – around 4 liters) the color of the flush solution draining is usually clear. The tip of the PA cannula must remain in the main PA and proximal to the bifurcation during the flushing. Both lungs should appear evenly blanched after the flush. After the completion of thoracic organ preservation infusions, the heart is excised first. The lungs remain cooled and gently ventilating in the chest for the few minutes required to excise the heart.

The pulmonary artery is transected at the site of the PA cannulation suture (half way between the pulmonary valve and the bifurcation). The aorta is transected. The IVC transection is completed, and the SVC is transected below the tied suture. The apex of the heart is elevated exposing the posterior left atrium and pulmonary veins. The left atrial incision is started at the atrioventricular groove (halfway between the left inferior pulmonary vein and the coronary sinus). Under direct vision inside the LA, the initial left atrial incision is extended and carried out parallel to the
atrioventricular groove toward the base of the left atrial appendage on the left and toward inferior pulmonary vein orifice on the right. The pulmonary vein orifices should be kept in sight to make sure that an acceptable cuff is maintained both on the left atrium on the heart and on the left atrium on the lung side (5-10 mm out of the pulmonary vein orifices).

The retrograde flush is initiated by infusing 1L of Perfadex® through an inflated Foley catheter (18 or 20 French) with its tip positioned in each individual pulmonary vein orifice sequentially (250ml for each vein).

After the retrograde flush is completed the mediastinum is dissected. Both inferior pulmonary ligaments are mobilized and divided by reaching behind the lung and retracting each lower lobe anteriorly and laterally gently. The dissection continues following the anterior wall of the esophagus until reaching the azygos vein on the right side and aorta on the left, both are transected. All mediastinal tissue is divided down to the level of the upper trachea which is then encircled. Prior to stapling the trachea, the lungs are recruited using a sustained airway pressure of 15-20 cmH₂O and a FiO₂ of 50%, the lungs should be about 75% inflated when they get stapled. To prevent contamination of the operative field with respiratory tract organisms, the proximal trachea is stapled twice with a TA-30 stapling device (Covidien, USA) and transected between the staple lines. The trachea should be transected as close to the larynx as possible. The lung block is removed. The lungs are examined for unexpected pathologic conditions, reviewing each lobe. The lungs are placed in a triple plastic bag containing 3 liters of Perfadex®, sitting on ice. The preservation bags are tied and the lungs are placed into a cooler surrounded by ice for transport.

Changes and improvements in lung preservation technique and preservation solutions itself have been crucial in making lung transplantation a reality and in improving post transplantation results. The foundation in organ preservation has been static cold preservation. Static cold preservation aims to slow down the metabolism reducing the oxygen consumption in an effort to maintain the organs.

Static cold preservation, that has been the foundation of organ preservation, it aims to slow cell metabolism and thus reduce consumption oxygen and other substrates in an attempt to prevent organ deterioration. This strategy reduces cellular activity in a non-selective way, including processes that lead to edema and cell damage. (Machuca, Cypel, and Keshavjee 2013; Hall et al. 1994)

Preservation solutions can be: intracellular or extracellular. Intracellular solutions (Wisconsin solution® and Euro-Collins®) have high potassium thereby prevent sodium from penetrating the cell. Furthermore they also include high molecular weight substances that cannot pass through the cell membrane and increase the extra-cellular osmotic pressure. Extracellular solutions (Perfadex® Low-Potassium Dextran and Czelsior®) have low potassium and high sodium. (de Perrot and Keshavjee 2004) See Table 4.

Table 4 Characteristics of Preservation Solutions

<table>
<thead>
<tr>
<th>COMPOSITION</th>
<th>SOLUTION</th>
</tr>
</thead>
</table>
| Intracellular| ↑ Potassium ↓ Sodium Wisconsin solution®
|              | Euro-Collins®                         |
| Extracellular| ↓ Potassium ↑ Sodium Perfadex® Low-Potassium Dextran
|              | Czelsior®                             |

The initial thought of using extracellular solution for lung preservation was presented by Fujimura and colleagues. (Fujimura et al. 1987) Keshavjee from the Toronto group published the improvement of
lung function through diminishing edema by adding dextran 40 as a rheologic and onctic agent to the extracellular low potassium solution. Dextran acts as osmotic agent retaining water in the intravascular space, it has shown to improve erythrocyte deformability and inhibit erythrocyte aggregation as well as acting as antithrombotic. (Keshavjee et al. 1989) The supplement of 1% glucose to the low potassium dextran solution was suggested by Date and colleagues in an attempt to sustain aerobic metabolism and maintain the cellular integrity. (Date et al. 1993) The current practice for most transplant centers in the world is the use low-potassium, dextran and glucose solutions, since their benefits have been shown in multiple studies. (Thabut et al. 2001; Fischer et al. 2001; Arnaoutakis et al. 2010)

During cold storage is important to avoid acidosis by maintaining the pH as close as possible to a normal pH. An increase of lactic acid and hydrogen ions affects normal cell activities and compromises energy production. Buffers are used to maintain the normal pH of the preservation solutions. Low potassium dextran solution (Perfadex®) has a pH of 5.5, this pH provides stability of the solution in storage for 3 years. Before using, 1 mmol/L of trometamol or tromethamine and 0.5-1 mmol/L of Ca²⁺ must be added in order to adjust the pH to 7.4.

Currently the recommendation is to use hypothermic solution to flush the lungs. Some beneficial effects of perfusion solution at higher temperatures (15-20°C) have been described experimentally although the studies have mainly been done in small animal. (Wang et al. 1993; Date et al. 1992; Albes et al. 1997)

Some experiments have shown a possible beneficial effect of maintaining slightly higher temperatures in the lung during cold storage (10°C), but other groups have not confirmed this. (Date et al. 1992; Kirk, Colquhoun, and Dark 1993; Kayano et al. 1999) This is also not easily or practically possible. Since the temperature of ice is 4°C, aiming for a preservation temperature in the range of 4-8°C is easily and safely achievable it is commonly used to simplify transport.
The retrograde flush during lung preservation has been demonstrated to be beneficial. Probably because it improves the distribution of the flush to vascular beds in the lung that may not have been completely flushed through the antegrade route. Retrograde flush also is able to remove clots from the pulmonary circulation, if present. (Struber et al. 2002)

Inflated lungs with oxygen during the cold ischemic period has been shown to be beneficial in numerous studies. (Baretti et al. 1995; Sakuma et al. 2000) The state of oxygenated ischemia is a known source of free oxygen radical production by reactions catalyzed by NADPH oxidase. (Al-Mehdi, Shuman, and Fisher 1997) Keeping the lungs inflated preserves the aerobic metabolism as well as the epithelial fluid transport and conserves the in the integrity of pulmonary surfactant. (de Perrot and Keshavjee 2004)

The vascular bed responds to atelectasis with vasoconstriction which translates into higher pulmonary vascular resistance. Hyperinflation of the lung is known to be also detrimental. Over-inflation during cold ischemia can lead to barotrauma and increased pulmonary capillary filtration coefficient that leads to edema. (Haniuda et al. 1996) If the donor lung is transported by air hyperinflation can occur inadvertently as a result of the low atmospheric pressure in flight, which results in gas expansion. Extra care must be taken to not fully inflate the lungs when the trachea is stapled so that there is room for some expansion at increased altitude. The ideal oxygen concentration during perfusion in unknown but most groups agree that a fraction between 30% and 50% can be used. (Hopkinson, Bhabra, and Hooper 1998; Padilla and Padilla 2004) Higher oxygenation concentrations can originate hyperoxidation and mitochondrial dysfunction by increasing lipid peroxidation. (Padilla and Padilla 2004)

It is generally safe to perform a gentle recruitment maneuver to ensure that the lung is fully expanded before flush perfusion and ventilate the lungs with peak pressure of 20 cm and a PEEP of 5 cm H₂O and FiO₂ of 50% during the perfusion period.
A dose of 500µg of prostaglandin E₁ (PGE₁) is given through the pulmonary artery during lung donor retrieval to induce pulmonary vasodilatation. PGE₁ has been shown to have anti-inflammatory properties by downregulating the cytokine production. Prostaglandin E₁ can improve pulmonary dynamic compliance after reperfusion. The current strategy for static preservation from the Toronto Lung Transplant Program strategy for static lung preservation is summarized in Table 5.

Table 5 Toronto Lung Transplant Program Technique for Lung Preservation

<table>
<thead>
<tr>
<th>Preservation solution</th>
<th>Low Potassium Dextran Solution (Perfadex® )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacological additives</td>
<td>Prostaglandin E₁, heparin, methylprednisolone</td>
</tr>
<tr>
<td>Anterograde flush (volume)</td>
<td>60 ml/kg</td>
</tr>
<tr>
<td>Retrograde flush (volume)</td>
<td>250 ml/pulmonary vein</td>
</tr>
<tr>
<td>Pressure during flush</td>
<td>30 cm H₂O (gravity drain by height above OR table)</td>
</tr>
<tr>
<td>Lung ventilation during flush</td>
<td>PEEP 5 cmH₂O and VT 8 ml/kg</td>
</tr>
<tr>
<td>Perfusion solution temperature</td>
<td>4-8°C</td>
</tr>
<tr>
<td>Oxygenation fraction</td>
<td>FiO₂ 50%</td>
</tr>
<tr>
<td>Airway pressure</td>
<td>15-20 cmH₂O (nearly complete expansion of lungs no overexpansion)</td>
</tr>
<tr>
<td>Storage temperature</td>
<td>4-8°C</td>
</tr>
</tbody>
</table>

Abbreviations: FiO₂, Fraction of inspired oxygen; PEEP, Positive end expiratory pressure; TV, tidal volume


1.3.3. Ex vivo Normothermic Lung Preservation Modified from: Mariscal, Andrea; Cypel, Marcelo; and Keshavjee, Shaf. (2017). Ex Vivo Lung Perfusion. Current Transplantation Reports. 4. 10.1007/s40472-017-0145-x. (Mariscal 2017)
The current conventional lung cold preservation strategy is focused in slowing the metabolic rate by using hypothermia. The majority of the damaging effects of ischemia/reperfusion are the consequence of chemical reactions. Hypothermia reduces the enzymatic activity that drive these processes, it reduces cellular oxygen and nutrient requirements as well as cellular metabolism. This way hypothermia is able to slow down cell death processes and maintain cell viability. However, hypothermia also reduces reparative and other beneficial organ functions, which avoids recovery of the lungs from injury.

One of the biggest limitations in lung transplantation is the shortage of donor lungs. In an attempt to overcome this imbalance, many lung transplant programs are currently using lungs that don’t comply with the established ideal criteria. Normothermic ex vivo lung perfusion (EVLP) has emerge as an ideal tool to assess and potentially recondition organs before transplantation while preserving the metabolic function and lung homeostasis.\(^\text{(Cypel et al. 2008)}\)

Around 80 years ago, Alexis Carrel and Charles Lindberg performed experiments using normothermic organ perfusion.\(^\text{(Carrel and Lindbergh 1935)}\) In an effort to perform a short functional evaluation of the lung in donors after cardiac death (DCD), Steen re-addressed the idea of the ex vivo lung perfusion. He was able to perform short-term perfusion using a buffered extracellular solution with a colloid osmotic pressure (Steen solution) combined with blood as the lung perfusate.\(^\text{(Steen et al. 2003; Steen et al. 2001; Steen et al. 1997)}\) Increased pulmonary vascular resistance and airway pressures that led to circuit-induced lung injury prevented Steen from being able to maintain the EVLP circuit running for longer than 120 min. The concept of extended EVLP (>12 h) was introduces by the Toronto group. They achieved this goal by modifying the EVLP technique using a lung protective strategy for perfusion and ventilation (Toronto EVLP technique).\(^\text{(Cypel et al. 2008)}\) Using Toronto EVLP technique the donor lungs are able to remain in a functional physiological
state for extended periods. In order to maintain this physiological stability the maximal flow was limited to 40% of cardiac output, the lower flow is able to perfuse the lung without producing hydrostatic edema secondary to mechanical stress. The maintenance of a positive left atrial (LA) pressure of 3-5 mmHg was found to open the distal veins preventing the vein from collapsing during decreases of flow at inspiration.\(^{(\text{Petak et al. 2002})}\) The closed atrial technique use by the Toronto group has proven to create a controlled positive LA pressure during EVLP, this controlled LA pressure lowers edema and superior lung physiology if compared with the open LA method.\(^{(\text{Linacre et al. 2016})}\) Toronto also encourages the use of a centrifugal pump to prevent circuit induced lung damage and the use of a protective ventilation. The perfusion used is an acellular perfusion which is logistically simpler and avoids the limited lifespan of red blood cells in the perfusion circuit. In summary the main achievement of the Toronto EVLP technique of maintaining donor lungs in a state where they are able to function in an optimal environment was accomplished by: using protective flow parameters to prevent mechanical stress; protective ventilation to avoid circuit-induced lung injury; and protective composition of the perfusate to achieve cell homeostasis. This extended EVLP technique provides a platform to not only assess but also for treatment delivery in the normothermic state.\(^{(\text{Cypel and Keshavjee 2015})}\)

There are three different protocols that have been published and tested in clinical trials: the Toronto EVLP Technique, The Lund (Vivoline, Lund, Sweden) technique and the Organ Care System Technique (OCSTM, Transmedics, Andover, MA, USA). All protocols share some characteristics and each one has distinctive features that characterize them. The differences can be seen in equipment, the ventilation, perfusion settings, and the perfusate composition. See table 6
### Table 6 Comparison Between EVLP Protocols

<table>
<thead>
<tr>
<th></th>
<th>LUND</th>
<th>TORONTO</th>
<th>OCS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Perfusate</strong></td>
<td>Extracellular + Albumin + Dextran 40 (Steen™)</td>
<td>Extracellular + Albumin + Dextran 40 (Steen™)</td>
<td>Low potassium dextran 40 (OCS™) + RBC hct 15-25%</td>
</tr>
<tr>
<td></td>
<td>Albumin + Dextran 40 (Steen™) + RBC hct 14%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Flow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type</strong></td>
<td>Continuous</td>
<td>Continuous</td>
<td>Pulsatile</td>
</tr>
<tr>
<td><strong>Initiation</strong></td>
<td>100 mL/min</td>
<td>10% of target</td>
<td>200 mL/min</td>
</tr>
<tr>
<td><strong>Target</strong></td>
<td>100% Cardiac output</td>
<td>40% Cardiac output</td>
<td>2-2.5 L/min</td>
</tr>
<tr>
<td><strong>Left Atrium</strong></td>
<td>Open</td>
<td>Closed</td>
<td>Open</td>
</tr>
<tr>
<td><strong>PAP</strong></td>
<td>$\leq 20\text{mmHg}$</td>
<td>$\leq 15\text{mmHg}$</td>
<td>$\leq 20\text{mmHg}$</td>
</tr>
<tr>
<td><strong>Ventilator</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Start temp</strong></td>
<td>32°C</td>
<td>32°C</td>
<td>34°C</td>
</tr>
<tr>
<td><strong>RR</strong></td>
<td>20/min</td>
<td>7/min</td>
<td>10/min</td>
</tr>
<tr>
<td><strong>TV</strong></td>
<td>5 mL/Kg donor BW</td>
<td>7 mL/Kg donor BW</td>
<td>6 mL/Kg donor BW</td>
</tr>
<tr>
<td><strong>PEEP</strong></td>
<td>50%</td>
<td>21%</td>
<td>21%</td>
</tr>
<tr>
<td><strong>FiO2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EVLP time</strong></td>
<td>2 h</td>
<td>4-6 h</td>
<td>Transport time</td>
</tr>
</tbody>
</table>

**Abbreviations:** RBC Red blood cells, PAP pulmonary artery pressure, RR respiratory rate, TV tidal volume, PEEP positive end expiratory pressure, FiO2 inspired fraction of oxygen, BW body weight. Modified from: Mariscal, Andrea; Cypel, Marcelo; and Keshavjee, Shaf. (2017). Ex Vivo Lung Perfusion. Current Transplantation Reports. 4. 10.1007/s40472-017-0145-x.

An extracellular solution supplemented with human albumin and dextran (Steen Solution™; XVIVO Perfusion, Denver CO, USA) is used by the Lund and the Toronto techniques. The albumin helps to maintain the colloid osmotic pressure. Steen Solution™ is also supplemented with dextran 40. Dextran protects the microcirculatory endothelium and is also able to inhibit platelet adhesion and coagulation. (Keshavjee et al. 1989) The Lund protocol includes supplementing the perfusion solution with red blood cells with a hematocrit of 14%.
The Toronto and Lund techniques use perfusate based on an extracellular solution with human albumin added (Steen Solution™; XVIVO Perfusion, Denver CO, USA), this solution maintains a colloid osmotic pressure; it also has dextran 40 to protect the microcirculatory endothelium and inhibit coagulation and platelet adhesion (Machuca and Cypel 2014; Van Raemdonck et al. 2015). The difference between them is that Lund also has red blood cells with a hematocrit of 14%. The perfusate in the OCSTM protocol is based on low potassium dextran 40 without additional human albumin: OCSTM Solution (Transmedics) or Perfadex™ (XVIVO Perfusion, Denver, CO) with red blood cells with a hematocrit of 15-25%.

The target flow during EVLP is set at 100% of cardiac output in the Lund protocol, 40% in the Toronto protocol and a fixed level of 2–2.5 l/min in the OCSTM protocol. The LA is left open to allow drainage of pulmonary effluent in the Lund and OCSTM protocols.

The Toronto Protocol uses a closed atrial cuff technique with a specially designed cannula to maintain a positive LA pressure between 3 and 5 mmHg and avoid the development of vascular stress injury. The PAP is maintained below 12 mmHg at targeted flow at all times in order to prevent the development of hydrostatic pulmonary edema (Broccard et al. 2002; Petak et al. 2009; Linacre et al. 2016).

In summary, the Toronto technique uses low potassium dextran solution with human albumin, target flow of 40% of the cardiac output, LA pressure between 3 and 5 mmHg and PAP below 12 mmHg. See Table 7.
1.3.3.1. **Ex vivo Perfusion for Clinical Evaluation**

In contrast to the conventional donor assessment, the ex vivo lung perfusion gives the unique opportunity of evaluating the function of the donor lungs over time. Following the Toronto strategy, physiological assessments are performed every hour. Before assessments, ventilation parameters are set to 10 ml/kg tidal volume, 10 breaths per minute, and FiO₂ 100%. Five minutes after changing the ventilation parameters, the delta pO₂ is calculated by analyzing the pre- and post-membrane perfusate gases. The other physiologic parameters that are recorded are: static compliance, dynamic compliance, PA pressure, LA pressure, peak airway pressure, and plateau pressure. After the assessment, the ventilation parameters are changed back to the initial pre-evaluation protective settings. The ventilation parameters are changed back to the maintenance parameters after the hourly evaluation. A lung x-ray is performed routinely as part of the 1st hour assessment of EVLP and then every two hours. Every 30 minutes, pulmonary recruitment maneuvers are performed by increasing the tidal volume by 100 cc with following by inspiratory hold maneuvers up to 25 cm H₂O (and a maximum of 15 cc/kg) for ten seconds.
According to the Toronto EVLP protocol EVLP runs for four to six hours and the decision to accept or reject the lungs is made after 3 hours of EVLP. At the 3 h point the functional data have been taken 3 times and the lung x-rays 2 times. The decision is made looking into the trend of the most important physiologic parameters (compliance, gas exchange, airway pressures). The worsening of those parameters usually mean development of edema secondary to lung injury. The first parameter to drop in an injured lung is the lung compliance followed by the delta pO$_2$ in perfusate. (Yeung, Cypel, et al. 2012) The specific acceptance criteria followed by the Toronto protocol are: PLAO$_2$/FiO$_2$ ratio $\geq$400 mmHg, stable or decreasing PA pressure, stable or decreasing airway pressure and stable or improved pulmonary compliance.
Table 8 Acceptance and rejecting EVLP parameters according to the Toronto EVLP Protocol

<table>
<thead>
<tr>
<th>ACCEPTANCE CRITERIA</th>
<th>REJECTION CRITERIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAO$_2$/FiO$_2$ ratio ≥ 400 mmHg</td>
<td>PLAO$_2$/FiO$_2$ ratio &lt; 400 mmHg</td>
</tr>
<tr>
<td>Stable or decreasing pulmonary artery pressure</td>
<td>&gt;15% deterioration on pulmonary artery pressure</td>
</tr>
<tr>
<td>Stable or decreasing peak airway pressures</td>
<td>&gt;15% deterioration on airway pressure</td>
</tr>
<tr>
<td>Stable or improving static + dynamic compliance</td>
<td>&gt;15% deterioration on static + dynamic compliance</td>
</tr>
</tbody>
</table>


1.3.3.2. Ex vivo Perfusion as a Platform for Organ Repair

Lungs can be injured by different mechanism including injury during brain dead or during the ICU stay (aspiration, ventilator induced lung injury, infection, edema, and atelectasis), these among other injuries can compromise the lung function and are responsible for the low utilization rate. During the last years, EVLP has proven to be a very useful platform to potentially repair some of this concerns, in different experimental and clinical studies. In order to be able to repair lungs, the EVLP needs to be running in a stable way during longer periods of time. (Cypel and Keshavjee 2016)

Pulmonary edema

Lung is probably the most susceptible organs in terms of developing edema during the pre-retrieval phase. One of the most common reasons to reject a donor lung is pulmonary edema. The edema is sometimes the consequence of the hemodynamic changes that take place during the development of brain death. The fluid overload during the ICU stay also may lead to pulmonary edema. EVLP itself is able to stimulate alveolar fluid clearance in the organ donor, reducing the edema in the lung. Some studies have shown the use of an inhaled β2-adrenoreceptor agonist (procaterol) given
during EVLP is able to reduce the lung injury in an animal DCD model. Inhaled procaterol elevated lung tissue cAMP levels and CFTR gene expression, decreased the wet to dry lung weight ratio and improved the lung function during 4 hours of EVLP. Beta2-agonist (terbutaline) has also been given during perfusion to increase edema fluid clearance in rejected donor lungs with acute lung injury and pulmonary edema.

**Gastric Aspiration**

One of the principal reasons for declining a donor lung is the concern of gastric aspiration. It is well known that gastric content aspiration leads to severe lung injury. The inhalation of gastric content can lead to aspiration pneumonitis and surfactant dysfunction. Different centers have studied the effect of EVLP treatment after aspiration. The Zurich group used a pig model were gastric juice was infused in 2008. Three years later in 2011 Meers et al. from Lueven, Belgium treated the lungs during EVLP using surfactant lavage. The injury was induced with 5-ml/kg administration of a betaine-HCl/pepsin mixture via a flexible bronchoscope. After injury, animals were randomly assigned (n = 6 in each group), to either receive saline lavage during EVLP (control); surfactant lavage EVLP (SL-EVLP); and surfactant lavage before harvest (SL-Pre); and a normal group (n = 4), with no lung injury. They published that EVLP surfactant lavage was able to improve the graft function of lungs injured by gastric acid aspiration. In 2014 Khalife-Hocquemiller and the group from Marie Lannelongue in Paris used another pig gastric acid aspiration model to examine the effects of surfactant administration during EVLP. The pigs were randomly assigned to three different groups: gastric acid induced injury + 4 h EVLP; gastric induce injury + surfactant lavage immediately before EVLP; saline infusion alone or saline infusion + by EVLP. The results showed that pigs in the group that received surfactant before
EVLP had improved functional parameters (PaO2, pulmonary vascular resistance, and apoptotic-cell percentage) and partially lower histologic severity score. (Khalife-Hocquemiller et al. 2014)

Nakajima et al. in 2015 showed in a gastric aspiration lung injury pig experiment that lung lavage followed by trans-bronchial administration of exogenous surfactant during EVLP improves the post-transplant lung function. After lung injury was induced with gastric juice (pH=3.0), pigs were ventilated for 6 hours, donor lungs were retrieved. Following 10 hours of cold ischemic time, lungs were randomized into 4 groups (n=5 each group): 1) no treatment (control); 2) lung lavage (LL); 3) surfactant administration (SF); 4) surfactant administration following lung lavage (SL). Lungs underwent EVLP for 6 hours. Following a 2 hour second cold ischemic time (Post EVLP), the left lung was transplanted and reperfused for 4 hours in all the groups. Surfactant administration alone significantly improved lung function during EVLP, but it was not enough to reduce inflammatory activity. Lung lavage followed by trans-bronchial administration of exogenous surfactant during EVLP provided superior post-transplant function (Nakajima et al. 2015)

**Infection**

Affecting up to 28% of intubated patients in the ICU, ventilator-associated pneumonia (VAP) is the most common nosocomial infection in the ICU. (Waters and Muscedere 2015; Porzecanski and Bowton 2006; Chastre 2006) Positive BAL cultures in the donor lung have been related to worse post-transplant outcomes. (Bonde et al. 2006; Avlonitis et al. 2003) According to recent studies, between 46% and 89% of the donor lungs have positive bacterial cultures in bronchoalveolar lavage (BAL). (Remund, Best, and Egan 2009; Nakajima et al. 2016) Infected lungs can be treated while in EVLP with the advantage of being able to give higher doses than normal without experiencing any systemic side effects. A study regarding 18 human donor lungs that were rejected for transplantation was published by Andreasson et al. The rejected lungs were treated with high-dose, broad-spectrum antibiotics during EVLP. From
the 18 rejected lungs, the microbial burden was reduced and 6 lungs were finally transplanted, all of the patients that ended up receiving the treated lungs survived to hospital discharge. (Andreasson et al. 2014) Nakajima et al. from the Toronto group also published the results from 15 human rejected donor lungs because of clinical suspicion of infection. After randomization (n=7 in each group), the treated group received meropenem 2 g, vancomycin 15 mg/kg and azithromycin 500mg in five cases, and meropenem 2 g, vancomycin 15 mg/kg and ciprofloxacin 400mg in three cases. They also demonstrated that the use of broad-spectrum antibiotics in the perfusate was able to reduce pathogen burden and endotoxin levels as well as lung injury. (Nakajima et al. 2016)

**Pulmonary Embolism**

During donor retrieval pulmonary embolism (PE) or infarctions are a common finding. (Ware et al. 2005) Pulmonary embolism has been associated with primary graft dysfunction and worse outcomes after transplant. (Oto et al. 2005)

Treatment for pulmonary embolism (pulmonary embolectomy and thrombolytic therapy) in donor lungs has been reported. (Frenia et al. 2005; Sareyyupoglu, Shigemura, and Toyoda 2011; Shihata et al. 2008) However the Toronto group published a case report of ex vivo therapy for thrombolysis and was able to successfully transplant the treated lungs. The donor had extensive acute PE and thrombolysis with alteplase was performed during EVLP. (Machuca et al. 2013) Subsequently there have been other clinical cases reporting the use of fibrinolytic agents (alteplase or urokinase) delivered during EVLP into the perfusate for donor PE, followed by successful lung transplantation. (Inci et al. 2014; Luc et al. 2015) In an attempt to reduce the pro-inflammatory environment IL-10 has been investigated as an anti-inflammatory cytokine. IL-10 is mainly produced by monocytes and lymphocytes. As an immune modulator it has shown to inactivate neutrophils and macrophages downregulating the production and secretion of pro-inflammatory cytokines. (Kaneda et al. 2006)
Gene Therapy

It is known that during the reperfusion phase of the transplant there is a pro-inflammatory cytokines release. Studies have demonstrated that cytokines including TNF-α IFN-γ IL-8, IL-10, IL-12, and IL-18 can be measured in lung tissue. (de Perrot et al. 2003) IL-10 gene therapy has shown to be beneficial by reducing lung injury and cell death after lung transplantation. (Fischer et al. 2003; Martins et al. 2004) Adenovirus have been used as vectors given their ability to achieve gene transfection in cells that are non-replicating. Adenovirus are able to transfect genes in an epichromosomal manner (without directly integrating into the host chromosomes). The comparison between intratracheal delivery of the adenoviral vector encoding IL-10 ex vivo versus in vivo was performed by Yeung et al. in 2012. They showed that lungs with perfused during 12 h had stable lung function. (Yeung, Wagnetz, et al. 2012)

Cypel et al. published a study were 10 human lungs that were found unsuitable for transplantation put in an EVLP circuit and perfused for 12h, 6 lungs were treated with adenovirus transfected with IL-10 (AdhIL-10). They showed that the AdhIL-10 group had significant lower pro-inflammatory cytokines and higher anti-inflammatory IL-10, AdhI-10 was also able to repair the alveolar-blood barrier integrity. (Cypel, Liu, et al. 2009)

Mesenchymal Stem Cell (MSC) - Based Therapy

MSC are stromal cells that are able to differentiate into different cell types such as osteoblasts, chondrocytes, myocytes, fibroblast and adipocytes. The definition of human MSCs established in 2006 by the International Society of Cellular Therapy states that: 1) Must be adherent to plastic under standard culture conditions. 2) As cell surface markers must express CD105, CD73 and CD90, and should not express CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules. 3) In vitro they must be able to differentiate to osteoblasts, chondroblasts, and
adipocytes. (Dominici et al. 2006) MSCs migrate to the sites of inflammation and generate an immunomodulatory and anti-inflammatory effect through cell-cell interactions between MSCs and lymphocytes or by the production of soluble factors. MSCs are able to inhibit T cell proliferation as well as inflammatory cytokine secretion. MSCs also control dendritic cells, B and T cells, neutrophils and prostaglandin (PGE$_2$). They secrete cytoprotective growth factors such as: keratinocyte growth factor, vascular endothelial growth factor, and hepatocyte growth factor, and angiopoietin-1 creating an endothelial barrier protective effect.

MSCs have been tested in different lung diseases models demonstrating that MSCs are able to ameliorate inflammation, inhibit apoptosis and reduce lung injury. (Matthay et al. 2010; Weiss et al. 2008; Weiss 2008; Ortiz et al. 2007) The delivery of MSCs in to the lungs can be done intravenous (systemic) or intratracheal (local). MSCs were given to human rejected lungs during EVLP by the University of California San Francisco group showing that MSCs treatment reduce lung edema and lowered inflammation by suppressing epithelial-specific growth factor production (Asmussen et al. 2014; McAuley et al. 2014; Lee et al. 2013; Lee et al. 2009; Gennai et al. 2015) (Matthay et al. 2010; McAuley et al. 2014) The Toronto group investigated the best route and dose of MSCs in a swine model, they found that intravascular delivery was more effective than airway delivery and that endothelial growth factor levels were higher in lung tissue and IL-8 levels in perfusate of MSC treated lungs. (Mordant et al. 2016)

1.4. Lung Epithelium and Endothelium

Lung alveolar epithelium represents a mechanical barrier that protects the lungs, it is formed by mainly 2 cell types: alveolar type I (ATI) cells (type I pneumocytes) and alveolar type II (ATII) cells (type II pneumocytes), that form a complete liner of the outlying part of the lung (the periphery). This lining plays a vital role in pulmonary homeostasis. The epithelium is also involved
in immune responses and it is in charge of regulating water and ions transport maintaining the alveolar fluid surface stability.\textsuperscript{(Guillot et al. 2013)}

About 90\% of the alveolar surface is covered by ATI cells their function is the gas exchange between alveoli and capillaries. ATI cells have a thin cytoplasm and a few mitochondria that makes this cells very sensitive to injury.\textsuperscript{(Herzog et al. 2008; Griffiths, Bonnet, and Janes 2005)} They have the ability to change their phenotype to ATII cells and proliferate.\textsuperscript{(Jain et al. 2015; Herzog et al. 2008)} ATI cells control cell proliferation, water and ions transport as well as peptide metabolism. ATI cells have ion channels and pumps for transcellular sodium transport and aquaporines that regulate water permeability.\textsuperscript{(Toczyłowska-Maminska and Dolowy 2012; Johnson et al. 2002)} ATI regulate macrophage function and participate in signaling pathways in peripheral lungs. ATI cells express toll-like receptors (TLRs) and respond to stimuli by producing of pro-inflammatory cytokines and modulate reactive oxygen species. ATI play an significant role regarding lung damage pathogenesis. Different stimuli, induce inflammatory damage to the lungs mediated by the receptor for advanced glycation end products (RAGE) which are expressed in the ATI cell high basal levels. RAGE stimulates NFκB that promotes the transcription of other pro-inflammatory factors, including TNFα or IL1β.\textsuperscript{(Li et al. 2014; Uchida et al. 2006; Nova, Skovierova, and Calkovska 2019)}

ATII cells represent 7\% of the alveolar epithelium (16\% of lung parenchymal cells). ATII cells are small and cuboid with many lamellar bodies and apical microvilli. Alveolar type II cells main function is the synthesis, excretion and recycling of proteins and lipids components from pulmonary surfactant. Surfactant is essential to maintain the respiratory surface area and it also has a role regarding local defense mechanism in the lung. Surfactant proteins (SPs) SP-A and SP-D, act as opsonins and regulate the function of inflammatory cells. SP-B and SP-C contribute to modulation of the lung immunity system \textsuperscript{(Mason 2006; Uhliarova et al. 2016; Mulugeta and Beers 2006)} ATII cells
participate in immunomodulation also by producing cytokines and growth factors as well as endogenous antimicrobial peptides (such as neutrophil α-defensins, β-defensins, kaelicidin hCAP18/LL-37). (Zissel et al. 2000) They also participate in the sodium transport. (Mason 2006; Nova, Skovierova, and Calkovska 2019)

Alveolar type II cells are multipotent cells with high plasticity and are able to self-regenerate and differentiate into ATI cells they are in charge of repairing damaged tissue. ATII cells express toll like receptor 4 (TLR4) located mainly in the intracellular space. TLR4 endocytosis is mediated by CD14. TLR activation induces the activation of NFκβ pathway and the secretion of IL6 IL8 and type 1 interferons. (Nova, Skovierova, and Calkovska 2019)

The alveolar epithelium is in charge of regulating water and ion exchanges. There are 2 principal transport pathways in the epithelium (the transcellular and the paracellular). The transcellular depends on the distribution of the ion channels and transports that are located in the apical and basolateral membrane where the Na+/K+-ATPase is located. The paracellular transport is located in the extracellular compartment between the lateral membranes of the epithelial cells, it is dependent on diffusion processes directed by chemical and electrochemical gradients all across the epithelial cell layer. The paracellular transport is controlled by protein complexes called tight junctions (TJ) formed at the lateral membrane’s apical side and are formed at cell-cell interfaces that seal the space. Tight junctions are located at the paracellular space between the endothelial cells and the epithelial cells.

Tight junctions seal the space but are also able to adjust permeability to ensure transport and exchange across the epithelium. It’s permeability is dependent on the protein composition. (Holter et al. 1986) Tight junctions are constituted of cytoplasmic proteins and transmembrane proteins including occludins, junctional adhesion molecules and claudins, which are the principal regulators of the TJs permeability called permselectivity. (Wittekindt 2017) Cytoplasmic proteins are situated at the
cytoplasmic plaque domain of the tight junctions and include ZOs, membrane-associated guanylate kinase inverted (MAGI), cingulin. ZO-1, a 220-kDa phosphoprotein, is one of the major cytoplasmic proteins of the tight junctions. It is a scaffolding protein that links transmembrane proteins, occludin and claudin, to F-actin in the cytoplasm.\textsuperscript{Citi and Cordenonsi 1998} A misfunction or loss of the TJs permeselctivity leads to a leakage of water and high molecular weight proteins into the airways resulting in alveolar edema and lung injury. The alveolar epithelium is adjacent to the endothelial layer. The vascular endothelium is a single-cell lining membrane that covers the internal wall of the vessels. It’s a barrier that separates the blood that is circulating, from the vascular smooth muscle cells but it also has a other important functions. Endothelial cells synthesize and release important factors which regulate vascular function that maintain a homeostatic balance. The pulmonary endothelium is a metabolically active surface, which provides hemostasis. It plays an important role during immunologic and inflammatory events by regulating vascular tone, and interacting with inflammatory cells. Any agent that causes pulmonary endothelial cell injury can lead to alterations in hemodynamics, hemofluidity, permeability, gas exchange, and intercellular signaling that finally result in the clinical picture of lung injury including edema, hypoxemia and pulmonary infiltrates in chest x ray.\textsuperscript{Pankratova et al. 2017} Alveolar macrophages (AM) are situated near the epithelial surface and endothelial cells. The interstitial space between these two kinds of cells contains fibroblasts.\textsuperscript{Heidemann et al. 2017}

1.5. Primary Graft Dysfunction

Primary graft dysfunction (PGD) is defined as an acute lung injury syndrome that occurs within the first 72 hours after lung transplantation, and represents the most serious early complication of this procedure\textsuperscript{Christie, Carby, et al. 2005; Porteous, Diamond, and Christie 2015; Christie et al. 1998}. PGD is similar in many
ways to Acute Respiratory Distress Syndrome (ARDS)\textsuperscript{(Christie et al. 2010)}, both clinically and pathologically. PGD is characterized by hypoxemia, pulmonary infiltrates on the chest x-ray, and lung edema.


The specific pathophysiologic mechanisms that cause PGD represent an active area of investigation. During lung transplantation, several factors influence early lung injury, including donor mechanisms of death, preservation of the donor lung, recipient surgery, and ischemic time between lung retrieval from the donor and reperfusion in the recipient. (Porteous, Diamond, and Christie 2015)

Processes that occur during ischemia-reperfusion (IR) are currently the most well-defined contributing factors for PGD.

1.6. Lung Ischemia Reperfusion Injury

Inflammation usually appears in response to infection, and it is essential for the elimination of pathogens as well as for tissue repair. Sterile inflammation appears during non-infectious circumstances including trauma, injury induced by chemicals, or ischemia. The activation of the innate immune system in this cases promotes inflammation. In lung transplantation, ischemia and successive reperfusion frequently leads to an acute, sterile inflammation after transplant called ischemia-reperfusion (IR) injury. The ischemia-reperfusion injury (IRI) itself activates innate immune responses and promotes a pro-inflammatory microenvironment that predisposes to the
development of lung allograft dysfunction, and directly contributes to morbidity and mortality after transplant. (de Perrot et al. 2003)

IR injury is a rapid and complex inflammatory response that entails endothelial and epithelial injury/dysfunction, innate immune responses activation including activation of alveolar macrophages that secrete neutrophil chemokines (CXCL1 and CXCL2) and pro-inflammatory cytokines, natural killer T cells that induce IL-17 secretion and neutrophil infiltration that release cytokines, ROS and form neutrophil extracellular traps (NETs). Most of these responses are initiated by rapid and strong generation of reactive oxygen species (ROS) by endothelial cells via NADPH oxidase that leads to cell/tissue injury.

Figure 4 Ischemia-Reperfusion Sterile Inflammation Response

Sterile inflammation activates the innate immune system. In lung transplantation, ischemia-reperfusion commonly leads to acute, sterile inflammation ischemia-reperfusion (IR) injury. IR injury is an inflammatory response that involves endothelial and epithelial dysfunction, innate immune responses activation involving activation of alveolar macrophages that secrete neutrophil chemokines (CXCL1 and CXCL2) and pro-inflammatory cytokines; natural killer T cells that induce IL-17 secretion and neutrophil infiltration that release cytokines; ROS; and neutrophil extracellular traps (NETs). These responses are mainly initiated by fast and strong generation of reactive oxygen species (ROS) by endothelial cells via NADPH oxidase that leads to cell/tissue injury.
Primary graft dysfunction begins even before transplant during cold ischemia. During ischemia, hypoxia induces pro-inflammatory immune cells, macrophages, and endothelial cells to generate reactive oxygen species (ROS) and induce the upregulation of nitric oxide synthases (NOS), cell surface molecules, nuclear factor κβ (NFκβ), and pro-inflammatory cytokines in the endothelium. (de Perrot et al. 2003; Kalogeris et al. 2012) Nicotinamide adenine dinucleotide phosphate (NADPH) gets also upregulated and plays an important role in ROS generation during ischemia and also after reperfusion. NADPH-oxidase is present in the plasma membrane of neutrophil, macrophages, and other phagocytic cells. (Dodd and Pearse 2000; Kalogeris et al. 2012) These agents can lead both directly or indirectly to increased pulmonary vascular resistance (PVR) by promoting changes in the microvasculature, and produce pulmonary edema. Pulmonary edema translates into poor gas exchange, increases the peak airway pressure, and causes elevation of the alveolar/arterial oxygen gradient. Humoral signaling is also activated. The most important humoral factors that have been related to ischemia reperfusion injury are complement, chemokines and cytokines. The activation of the complement increases the damage to the lungs by causing direct lysis thorough generation of attack complexes in plasma and by further recruiting and activating neutrophils and macrophages. (Kalogeris et al. 2012; Eltzschig and Eckle 2011)

The reperfusion injury occurs in two phases. During the initial phase (thirty minutes after reperfusion), activated macrophages release IL-8, IL-12, IL-18, TNF-α, and platelet activating factor (PAF). The second phase, which peaks about four hours later, depends on ROS, IL-8, PAF, and TNF-α released by activated neutrophils. Neutrophils also release proteolytic enzymes that can increase the lung tissue microvascular permeability. Adhesion molecules, such as CD18, endothelial intercellular adhesion molecule-1, and endothelial P-selectin, are also upregulated and affect the microvascular permeability. These agents can lead both directly and indirectly, via
changes in the microvasculature, to increased pulmonary vascular resistance (PVR), which in turn causes pulmonary edema. Pulmonary edema translates into poor gas exchange, increases the peak airway pressure, and causes an elevation in the alveolar-arterial oxygen gradient. Humoral signaling is also activated, increasing the damage in both lungs\textsuperscript{(Fiser et al. 2001)} Superoxide production increases during reperfusion, if there is no effective dismutation of the superoxide anion it reacts with NO and forms peroxynitrate that is known to react with the cellular proteins and lipids to induce organ dysfunction.\textsuperscript{(Yurdakan et al. 2012)}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Ischemia Reperfusion Injury}
\end{figure}

During ischemia there is a lack of blood flow that leads to loss of mechanotransduction in the epithelium. Stimulating macrophages and endothelial cells to generate reactive oxygen species (ROS), it also increases NADPH, nuclear factor-κB (NF-κB), nitric oxide synthases (NOS), and cell surface molecules. After reperfusion, ROS and cytokines activate neutrophils. Neutrophils, platelets and nitric oxide (NO) signaling pathways, induce vascular damage that contributes to the development of pulmonary edema and cell apoptosis developing organ dysfunction.
Activated neutrophils play a key role in IRI. Neutrophils increase the expression of CD18 and CD11b and adhere to the endothelium with specific ligands (intercellular adhesion molecule-1 and P-selectin). Neutrophils increase the release of chemotactic agents and pro-inflammatory cytokines, and prolong oxidative stress in endothelial cells. They also release proteases, such as neutrophil elastase (NE).

NE is a serine proteinase that can be secreted by neutrophils and macrophages during inflammation.\textsuperscript{(Travis and Salvesen 1983)} NE can act both intracellular as a component of the innate immune system, and extracellular. Intracellular neutrophil elastase has a strong antimicrobial activity against gram-negative bacteria, spirochaetes and fungi.\textsuperscript{(Chua and Laurent 2006)} It’s best known extracellular function is the digestion of connective tissue but NE is capable of digesting all types of matrix protein (collagen, fibronectin, proteoglycans, heparin and fibrin). NE has other biological functions, it modulates inflammation and acts as a secretagogue for cytokines, mucin, glycosaminoglycans.\textsuperscript{(Chua and Laurent 2006; Travis 1988; Watorek et al. 1988)}

Studies have shown that NE can play an important role in the pathophysiology of acute lung injury.\textsuperscript{(Kawabata, Hagio, and Matsuoka 2002; Tamakuma et al. 2004)} At the sites of inflammation NE, appears to remain active probably because of an imbalance between NE and its protease inhibitors. In lung, the proteolytic activity of NE can lead to changes that are related to the pathophysiology of acute lung injury. Neutrophil elastase disturbs the lung permeability barrier and induces the release of pro-inflammatory cytokines such as interleukin 6 and 8.\textsuperscript{(Kawabata, Hagio, and Matsuoka 2002)}

Cell death occurs during IR-induced lung injury. Apoptosis has been historically considered the principal type of cell death in this setting. Apoptosis can happen through two different pathways, for the “extrinsic” pathway receptors Fas, TRAIL and TNFα and their ligands get activated this recruits death domain proteins in to the death signaling complex that activates caspase 8 (as an
initiator caspase); caspase 8 activates caspase 3 and other effector caspases that trigger enzymes that are responsible of the externalization of the phosphatidylserine, the condensation of the nucleus and finally the fragmentation of the DNA. The second pathway or “intrinsic” is triggered by intracellular stress or cytotoxic insult that induces the translocation of the Bcl-2 family proteins that induces mitochondrial membrane permeabilization that translates into the release of cytochrome c, Smac/DIABLO, endonuclease-G and other pro-apoptotic proteins. Cytochrome C binds with apoptotic protein activating factor 1 forming an “apoptosome” that activates caspase-9 and caspase-3. Both pathways interrelate in some situations in caspase-8.

More apoptotic cells are found in lungs that had cold ischemic times less than 12 hours and there are studies have questioned if apoptosis influences lung function after transplant.

A necrotic cell is defined by its morphologic changes characterized by swelling of the cells and its organelles, lack of nuclear fragmentation, rupture of the membrane and leakage of intracellular substance. All these are irreversible changes that occur after cell death. Necrosis in contrast to apoptosis is found in transplanted lungs with longer cold ischemic times (18-24 hours) and it correlates with bad lung function after transplant.

Previous studies have found other cell death pathways that are implicated in the process. This include caspase-independent cell death mediated by other proteases that can be released by lysosomes; oncosis that occurs when ATP generation is attenuated and cells begin to swell increasing the membrane permeability; and autophagy that degrades cellular components in vacuoles.
Endothelin-1 (ET-1) is one of the endothelium-derived relaxing factor (EDRF) discovered in the late 80’s. It is produced in the endothelial cells and secreted mainly to the basal part of the endothelium. ET-1 is able to induce vasoconstriction as well as relaxation. ET-1 produces vasoconstriction acting on the smooth muscles and also relaxation by releasing prostacycline and nitric oxide. It acts on the near endothelial cells in an autocrine and paracrine way. The state of the endothelium conditions the endothelin and nitric oxide production. Oxidized low-density lipoprotein (oxLDL) has shown to induce endothelial dysfunction by generating reactive oxygen species (ROS), and activated nuclear factor κB (NFκB), resulting in down-regulation of NO and up-regulation of ET-1.

There is currently no effective therapy for the prevention or treatment of PGD, and there is an urgent need to find new strategies to decrease or completely prevent the processes leading to IRI. (Diamond et al. 2017) In order to effectively study the mechanisms causing IR lung injury, and investigate potential therapeutics to prevent PGD, it is essential to have a clinically relevant model that closely mimics what occurs throughout lung transplantation, to test clinically relevant therapeutics. In the present study, we focus on alpha 1 antitrypsin

1.7. Alpha 1 Antitrypsin

1.7.1. Background

Human alpha-1 antitrypsin (A1AT) is also known as alpha 1 proteinase inhibitor and serine protease inhibitor, group A, member I (SERPINA I). It is a circulating glycoprotein, its principal function is to inhibit protein elastase as well as other serine proteases in blood and in tissue. A1AT is the most abundant of the protease inhibitors. The proteases inhibitory function of A1AT was revealed by Fermi and Pernossi after initial researches performed by others at the end of the 19th
century. In 1955 Herman Shultze isolated the protein that was responsible for bloods antiprotease activity and called it α-1 antitrypsin by its location in the α-1 band of the globulins and anti-trypsin because of its ability to inhibit the pancreatic enzyme trypsin. (Blanco 2017)

The A1AT protein is composed from a 394 amino acids chain with three N asparaginyl-linked complex type carbohydrate side chains. Of the molecule 30% is in the form of α-helices (9 alpha helices A-I), and 40% is in form of β-sheets (3 beta-sheets A-C). On the outside surface of one half of the elongated structure, the carbohydrate chains are attached to residues Asn46, Asn83, and Asn247. On the opposite side there is the exposed loop that has the reactive center (Met358, Ser359).

The structure of the A1AT stresses the active site loop, fitting the Met358, Ser359 tip close into the reactive pocket of neutrophil elastase where it can be held. (Crystal et al. 1989)

Alpha-1 antitrypsin (A1AT) has been subject of extensive research for the last 50 years. It is a medium-sized 53-kDa glycoprotein that is part of the serine protease inhibitor superfamily. Humans produce 34 mg/Kg/day, this translates into normal human serum concentrations of 0.9-1.75g/L, and the half-life is 5 days. (Wanner, Sandhaus, and SpringerLink (Online service))

This hydro-soluble and tissue-diffusible molecule is mainly synthesized by the liver (80%), but is also produced by pulmonary alveolar cells and certain immune cells such as monocyte-derived macrophages, monocyte derived dendritic cells, alveolar macrophages, and neutrophils (especially during activation). (van’t Wout et al. 2012; Clemmensen et al. 2011) From plasma, 80% diffuses into the interstitial tissues, 5-10% and gets to biological fluids, including alveolar fluid where A1AT concentrations are around 0.1-0.3 g/L. After production, A1AT uptake happens mainly mediated via clathrin, this results in transcytosis across the endothelial cell layer.

The specific substrate of A1AT is elastase mainly from neutrophils (25-fold greater than for the interaction with any other proteases) (Crystal et al. 1989; Travis and Salvesen 1983), but A1AT is also able to
neutralize proteinase-3, cathepsin G and myeloperoxidase from neutrophils; as well as mast cells chymase and tryptase and granzyme-B from T lymphocytes; pancreatic trypsin; kallikreins 7 and 14; and some of the serine proteinases from the coagulation cascade.

Neutrophil elastase (NE) is a 29kDa single chain, 220 residue enzyme with two carbohydrate side chains. It is non-specific and is able to act not only against elastin, but also many other connective tissue proteins including collagens type I and III from the alveolar interstitium, the protein portion of proteoglycans, type IV collagen and laminin from the basement membranes. Elastase is deposited in the azurophilic granules of the neutrophils and it is released either when the neutrophil gets activated or when it is disintegrated.

A1AT is confers over 90% of the protection against proteolytic capacity of neutrophil elastase in the lower respiratory tract. It is a very good inhibitor of neutrophil elastase. The only “weak point” of the A1AT against the neutrophil elastase is that Met_358 is susceptible to oxidation, when it gets oxidized the association rate is reduced up to 2000 fold. (Crystal et al. 1989)

A1AT is an acute phase protein; it can increase in serum concentrations to 4 times higher than homeostasis during an inflammatory response or infection. A1AT inhibits excess of proteolytic enzymes like neutrophil elastase and other proteases so they do not accumulate and break down normal tissue, and maintains a balance between tissue protease and anti-protease activity. (Wanner, Arce, and Pardee 2012)

To accomplish its function A1AT undergoes a conformational change where the substrate protein associates with a loop region on A1AT, causing that loop to become ordered as a beta strand (Whisstock and Bottomley 2008). Trypsin is inhibited as the substrate when a covalent bond is formed to A1AT through the beta region. Once bound covalently, the stability of the A1AT complex goes up, working as an effective molecular trap. (Whisstock and Bottomley 2008) Upon binding, NE cleaves the
A1AT reactive center loop that releases energy and results in a conformational change in which NE is flipped to the opposite end of the A1AT molecule. In the process, NE gets inactivated. The resulting A1AT–NE complex is then recognized by hepatic receptors and cleared from circulation. By forming the 1:1 complex, the A1AT molecule acts like a “suicidal protein”.(Wanner, Sandhaus, and SpringerLink (Online service))

Extraordinarily A1AT has shown to play a very important role in the protease-antiprotease homeostasis, allowing enough neutrophil elastase needed for host defense at the same time that it provides anti-elastase function to protect the host from further injury.(Gattman et al. 2015)

1.7.2. Alpha 1 Antitrypsin Deficiency

The inherited A1AT deficiency was described as a model of genetic disease first in 1963. This deficiency results in loss of function of the plasma protein (loss of lung elasticity and emphysema), as well as gain of function by liver accumulation (cirrhosis). (Wanner 2009)

The A1AT gene locus is located in the long arm of chromosome 14 (14q31-32.3). The gene has seven exons (3 of them: Iₐ, Iₖ, and Iₐ, contain sequences found in the A1AT mRNA but do not encode for the protein), and six introns. The active site of the mature protein gives specificity to the functional domain of the inhibitor and is encoded in the seventh exon (exon V).(Perlmutter and Pierce 1989)

The A1AT gene is known to be polymorphic with more than 75 alleles classified in four categories subject on the A1AT status in plasma:

1. Normal: A1AT levels around 0.9-1.75g/L and normal function.
2. Deficient: A1AT levels < 35% of the normal (0.9-1.75g/L).
The alleles nomenclature is based on letters that relate to the position of the A1AT in plasma between pH of 4.2 and 4.9 when seen in isoelectric focusing. The most common normal variants migrate to the middle: “M-family” alleles. The deficiency variants first described are the ones that migrate near pH 4.5 and were called “Z”. The A1AT serum phenotype is determined by the expression of the two parental alleles (the two parental genes are expressed in a codominant manner). The phenotype establishes the serum concentration of A1AT. Inheritance of any combinations of normal, deficient or null alleles determines the risk for developing disease (emphysema, liver disease or both). Four alleles (M1(Ala$^{213}$), MI(Val$^{213}$), M2 and M3) exemplify more than 95% of the normal known A1AT variants. M1(Ala$^{213}$) is the most common among Caucasians. There have been at least other 42 more rare normal A1AT variants identified. The classic deficiency allele Z was first described, the second more common deficiency allele is S. The coding exons for Z gene diverge from the normal M1(Ala$^{213}$) by only one base substitution, this substitution affects the ability of the cell to secrete A1AT, translating into lower A1AT plasma levels. In the Z homozygotes patients, A1AT mRNA levels and A1ATmRNA translation and glycosylation of the fresh synthetized A1AT are normal, but after the glycosylation, the Z A1AT aberrant polymerised molecules aggregate and accumulate in the endoplasmic reticulum of the hepatocytes and this results in lower levels of circulating A1AT. The A1AT molecule doesn’t function properly at inhibiting neutrophil elastase, because of the Z molecule having lower association rate and also being unstable after binding the NE (the elastase cleaves the Met$^{358}$-Ser$^{359}$ bond and releases de elastase).

The S allele is more common than Z. The exons from the S gene differ from the normal M1(Val$^{213}$) gene in one base (substitution of Glu$^{364}$ to Val. The S homozygotes (PiSS) have lower A1AT levels, but in still in the protective range (relative deficiency). PiSZ heterozygotes have borderline
low serum A1AT levels and some of them develop emphysema. In this individuals A1AT mRNA transcripts are normal in levels and length, but the secretion is around 40% lower than the M-homozygotes. The A1AT synthesized by PiSS individuals does not accumulate in the liver.

The null gene is an A1AT gene that doesn’t code for A1AT. Null homozygote patients have no circulating A1AT and develop emphysema, but no liver disease.

There is just one well described dysfunctional A1AT variant (A1AT_{Pittsburgh}). This A1AT molecule has a single amino acid substitution at the A1AT active inhibitory place (Met^{358} to Arg), this confers the A1AT molecule a very similar structure to antithrombin III and makes patients develop severe bleeding disorders.

1.7.3. A1AT Augmentation Treatment

The most specific therapeutic approach for patients with A1AT deficiency is purified human plasma A1AT preparations as augmentation therapy. It was first approved for patients with homozygote A1AT deficiency (PiZZ) or combination of null or other rare Z alleles, presenting with emphysema and levels of A1AT (0.5 g/l) by the United State Food and Drug Administration in 1987. The main aim is to increase serum and lung interstitial levels above 0.5 g/l in order to inhibit the neutrophil elastase activity, protecting against proteolytic destruction of alveoli and development of emphysema. Wewer confirmed in 1987 that purified human A1AT intravenous therapy was safe and was able to raise the A1AT levels (biochemical efficacy).\cite{Wewers_1987} The inhalation delivery of A1AT has been tested but it is still experimental.

The A1AT replacement dosing regimen is based on the biochemical efficacy criteria (increase A1AT plasma levels above the protective threshold of 0.5 g/l). This protective level was determined with epidemiological studies that have shown that A1AT levels below 50 mg/dl are associated with an increased risk of emphysema, levels between 0.5-8 g/l. This levels can be
achieved with weekly infusions of 60 mg/Kg of human purified A1AT. The weekly regimen was chosen based on the A1AT half-life.\textsuperscript{(American Thoracic and European Respiratory 2003; Wewers et al. 1987)} The inconvenience of a weekly infusion can be overcome with alternative schedules. The twice weekly schedule (120 mg/kg of A1AT) has shown to increase A1AT levels in 41\%, but the levels remain high just for one week, according to studies. The monthly infusion (240 mg/Kg) has also been tested, showing a gradual increase over time that lasted for an average of 25 days after each dose, although it was tested in a small number of emphysema patients \textsuperscript{(Hubbard et al. 1988)}. There are essentially seven different purified human A1AT preparations currently available (See Table 9).

\textbf{Table 9 Human Purified A1AT Preparations}

<table>
<thead>
<tr>
<th>NAME</th>
<th>MANUFACTURER</th>
<th>SOURCE</th>
<th>PURIFICATION PROCESS</th>
<th>COUNTRY APPROVED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolastin</td>
<td>Grifols (Spain)</td>
<td>Pooled human plasma</td>
<td>Pasteurisation</td>
<td>Austria, Belgium, Denmark, Finland, Germany, Greece,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ireland, Italy, Netherlands, Norway, Poland, Portugal,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spain, Sweden, Switzerland</td>
</tr>
<tr>
<td>Prolastin C</td>
<td>Grifols (Spain)</td>
<td>Pooled human plasma</td>
<td>Solvent purification + nanofiltration</td>
<td>USA, Canada, Argentina, Colombia</td>
</tr>
<tr>
<td>Trypsone</td>
<td>Grifols (Spain)</td>
<td>Pooled human plasma</td>
<td>Solvent purification + nanofiltration</td>
<td>Spain, Argentina, Brazil, Chile, Mexico</td>
</tr>
<tr>
<td>Aralast NP</td>
<td>Baxter (USA)</td>
<td>Pooled human plasma</td>
<td>Solvent purification + nanofiltration</td>
<td>USA</td>
</tr>
<tr>
<td>Zemaira</td>
<td>CSL Behring (USA)</td>
<td>Pooled human plasma</td>
<td>Pasteurisation + nanofiltration</td>
<td>USA, Brazil</td>
</tr>
<tr>
<td>Glassia</td>
<td>Kamada (Israel)</td>
<td>Pooled human plasma</td>
<td>Solvent purification + nanofiltration</td>
<td>USA, Brazil</td>
</tr>
<tr>
<td>Alfalastin</td>
<td>LFB (France)</td>
<td>Pooled human plasma</td>
<td>Pasteurisation</td>
<td>France</td>
</tr>
</tbody>
</table>

A1AT augmentation therapy has shown to be safe. There are very few and generally mild side effects reported. There are mainly two reports (one European and one from the United States) that published the A1AT side effects.

In 1998 from a total of 58,000 A1AT infusions Wencker and colleagues in Germany, reported 124 side effects (from 65 patients). Almost all of the adverse reactions were related to intravenous administration of proteins, including: fever or chills, urticaria, nausea, vomiting or fatigue. Some patients (17) described dyspnea that was believed to be related to the protein content in the intravenous solution. Only 5 severe event were reported, four patients had an anaphylactic reaction and one patient showed exacerbation of a congestive heart failure that ended up recovering. (Wencker et al. 1998)

Stoller and colleagues in the United States used the National Heart, Lung and Blood Institute (NHLBI) Registry to review the side effects from A1AT augmentation therapy. The overall rate of reported adverse events was 0.02 per patient/month, 10% of them were considered mild, 76% moderate and 9% severe. The most usual ones were headache, dizziness, nausea and dyspnea. Patients that received weekly infusions had a higher rate of side effect. No anaphylactic reactions were reported. (Stoller et al. 2003) In summary A1AT seems to be a safe with infrequent and generally well tolerated side effects. (Petrache, Hajjar, and Campos 2009)

Due to the infrequency of the disease, the studies regarding A1AT therapy in deficient patients remain usually under powered. For this reason most of the data supporting clinical efficacy come from indirect data from observational studies. (Survival and FEV1 decline in individuals with severe deficiency of alpha1-

The clinical efficacy has been tested in few randomized placebo-controlled studies. One of the most recent study was published in 2015 and showed evidence that purified A1AT augmentation therapy slowed progression of emphysema, lowering annual rate of lung density decline (they measured lung density with computed tomography and total lung capacity). (Chapman et al. 2015) A1AT therapy replacement needs to be further tested using other placebo-controlled studies and looking for more conventionally accepted criteria to study drug efficacy in patients with emphysema.

1.7.4. Other Effects of Alpha 1 Antitrypsin

A1AT has been shown to have other potential anti-inflammatory and immunomodulatory effects in different models. See table 10.
Table 10 Other effects of A1AT in different models

<table>
<thead>
<tr>
<th>DISEASE MODEL</th>
<th>DOSE</th>
<th>EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graft-versus-host disease (Marcondes et al. 2014)</td>
<td>A1AT (Glassia®, Kamada Ltd) 3 mg intraperitoneally</td>
<td>Promoted expansion of DCs, Tregs, and NK cells, decreased proinflammatory and enhanced antiinflammatory (IL-10, IL-1Ra) cytokines, increased survival.</td>
</tr>
<tr>
<td>Islet transplantation</td>
<td>Exogenous Transgenic (plasmid-derived)</td>
<td>Improved islet survival, reduced immune cell infiltration, increased insulin content, elevated intra graft VEGF transcript levels, improved immune tolerance (smDC), elevated IL-1Ra levels.</td>
</tr>
<tr>
<td>Inflammatory bowel disease (Collins et al. 2013; Janciauskiene and Welte 2016)</td>
<td>Aralast® (Baxter) Prolastin® C (Grifols) intraperitoneal 2 mg/24h</td>
<td>Reverses intestinal lesions, improves epithelial barrier function, attenuates proinflammatory cytokine production and inflammatory cell infiltration, reduces injury.</td>
</tr>
<tr>
<td>Collagen-induced arthritis (Grimstein et al. 2011; Grimstein et al. 2010)</td>
<td>Human A1AT 0.5mg intraperitoneal injection, 2/week or recombinant adeno-associated vector expressing hAAT</td>
<td>Lowers disease score, delays disease onset. Suppression of arthritis development</td>
</tr>
<tr>
<td>Acute liver failure (Jediche et al. 2014)</td>
<td>Purified human AAT (Prolastin®, Grifols) or oxidize A1AT 0.5 mg/mouse</td>
<td>Decreases serum TNF-a levels, decreases liver cell apoptosis, and prolongs survival.</td>
</tr>
<tr>
<td>Diabetes (Weir et al. 2018; Gottlieb et al. 2014; Ma et al. 2010)</td>
<td>Purified human AAT (Aralast, Baxter) 45-90 mg/kg/wk IV; (Prolastin®) 2 mg intradermal</td>
<td>Reduces b cell injury, improves insulin release, inhibits inflammatory cytokine production. A1AT is safe and suppresses NFκβ</td>
</tr>
<tr>
<td>Renal ischemia–reperfusion injury (Maicas et al. 2017)</td>
<td>Prolastin® 0.5 mg at reperfusion or 2 h after reperfusion. 80 mg/kg Prolastin® daily intraperitoneal</td>
<td>Decreases TNF-a expression and neutrophil influx.</td>
</tr>
<tr>
<td>Lung challenge with inflammatory stimuli (Lockett et al. 2013; Libert et al. 1996)</td>
<td></td>
<td>Selective inhibition of TNF-α–induced-self amplification, which may assist the vasculature in the resolution of chronic inflammation Improves lung function, reduces bronchoalveolar neutrophils counts, decreases IL-1b, TNF-a, and IL-8, decreases TLR2/4 expression.</td>
</tr>
</tbody>
</table>

endothelial cell apoptosis (Petrache, Fijalkowska, Medler, et al. 2006; Serban and Petrache 2016), and inflammatory signaling (Ehlers 2014). For A1AT to be able to fully exhort these protective effects in the endothelial cells it has to get intracellular (Lockett et al. 2014). Latest reports have shown that A1AT gets intracellular through endothelial cell endocytosis followed by the transcytosis and secretion (Gao et al. 2014) at the basolateral surface, then A1AT gets internalized by epithelial cells. (Lockett 2017)

Recent studies have shown that A1AT may have other biological functions, such as the inhibition of inflammation and apoptosis in vitro and in vivo. Our group demonstrated the anti-inflammatory and antiapoptotic effect of A1AT with an ischemia-reperfusion cell culture model. (Gao et al. 2014) We have also tested the effect of A1AT in a rat lung transplant model as well as in a non-survival pig left lung transplant setting where the allograft showed better lung physiological function and better oxygenation in the A1AT treated group. (Iskender et al. 2016) Studies have shown that A1AT has anti-apoptotic effects in lung alveolar cells and lung endothelial cells through inhibition of caspase-3. (Petrache, Fijalkowska, Medler, et al. 2006; Petrache, Fijalkowska, Zhen, et al. 2006) A1AT is internalized by lung endothelial cells it then binds to active caspase-3 and inhibits apoptosis. (Petrache, Fijalkowska, Medler, et al. 2006; Petrache, Fijalkowska, Zhen, et al. 2006) We recently reported the beneficial effect of A1AT administered during EVLP to improve donor lung quality after prolonged hypothermic preservation. (Lin H 2017)

A1AT has also shown protective effects against hepatic, renal, and myocardial IR injury. (Toldo et al. 2011; Gao et al. 2014; Maicas et al. 2017; Jedicke et al. 2014; Iskender et al. 2016)

Angiopoietin-like protein 4 (Angptl4), is one of the known target genes of peroxisome proliferator-activated receptor (PPAR) γ. Recent studies suggest that Angptl4 plays a role in inflammation (Lichtenstein et al. 2010). The expression of Angptl4 is controlled by peroxisome proliferator-activated receptors (PPARs). PPARs are a group of nuclear receptor proteins that
function as transcription factors regulating the expression of genes. Free fatty acids (FAs), which activate the lipid-sensing peroxisome proliferator-activated receptors (PPARs) α, β, and γ, are able to stimulate Angptl4 expression directly. (Frenzel et al. 2015) Recent investigations have shown that A1AT has the ability to upregulate both expression and release of Angptl4 in monocytes and in lung endothelial cells. According to a study published in 2015, A1AT binds fatty acids (FA) and only FA-bound forms of A1AT induce Angptl4. (Frenzel et al. 2015)

**Figure 6 Ischemia-reperfusion induced Lung Injury**

Mechanism by which A1AT could decrease ischemia reperfusion induced injury are circled: Inhibition of inflammation protease degradation, inflammatory signaling, macrophage and endothelial cell activation, ROS production, apoptosis, vascular damage, and inhibition of neutrophil elastase

**CONCLUSION**

A1AT has been subject of extensive research over the past 50 years. Recent studies have demonstrated that A1AT can have anti-inflammatory and immunomodulatory properties in different settings. Our group has been studying the potential benefit of using human A1AT before lung transplantation indifferent cell cultures and animal models. The first studies were done in an
ischemia-reperfusion lung epithelial cell culture, followed by a rat ischemia-reperfusion model and a non-survival left single transplant model in pigs. All this experiments were able to demonstrate some anti-inflammatory effect by lowering different pro-inflammatory cytokines and improving lung function after transplantation in different degrees. A1AT has proven to be a safe and well tolerated drug when used to treat A1AT deficiency, but the safety of the drug in high doses in a transplant hasn’t been tested yet.

During these PhD studies we will address the safety of high doses of A1AT in a transplant setting using a pig lung transplant survival model and we will test the efficacy of human A1AT on human lungs subjected to different types of injuries.
Chapter 2. Rationale, Hypothesis and Objectives
2.1. Rationale

Despite more than 30 years since the first lung transplant was performed and all the advancements in the field of transplantation, the utilization rate of offered donor lungs remains low. With a growing wait list, this translates into shortage of donors and around 15% of patients dying while waiting for lungs. According to UNOS only 1 in every 5 donor lungs are finally used for transplantation. (Klein et al. 2010; Kim et al. 2014; Singh et al. 2019) After the initial assessment most of the lungs are considered to be too injured to be safely used, and even the most aggressive lung transplant centers end up using less than 40% of the offered lungs. The development of ex vivo lung perfusion opened the door not only to a deeper evaluation of the offered donor lungs, but also to the possibility of repairing the lungs before transplant. Expanding the number of donor lung organs through the use of target therapies has the potential to improve the disparity without compromising recipient outcomes.

The use of A1AT as a strategy to decrease ischemia reperfusion injury has been studied in the Latner Thoracic Surgery Laboratories by different lab members for the last 8 years. This research builds upon the previous studies and findings where A1AT seems to be a promising alternative to reduce lung injury after transplantation. An IR epithelial cell culture model showed that A1AT was able to prevent cell morphology change after 18 hours of cold ischemic time and 4 hours of reperfusion; A1AT also reduced induced cell death via caspase-3 inhibition and reduced pro-inflammatory cytokine release in vitro including IL-6 and IL-8; A1AT additionally inhibited IR-induced loss of cell viability. (Gao et al. 2014) In a rat IR model, our group was able to show that after 90 minutes of ischemia and 120 minutes of reperfusion, lung oxygenation function of rats that received A1AT was significantly better compared with those in the control group. A1AT treatment also reduced the lung injury score, reduced neutrophil infiltration in the lung tissue and IR-induced
lung epithelial apoptosis. A1AT treatment significantly reduced plasma levels of IL-1α and TNFα. To further test the effect of A1AT in a more relevant in vivo model, we tested the effect of A1AT on IRI using a pig left single transplant model with 4 hours of reperfusion. Treatment with A1AT significantly improved allograft gas exchange, wet-to-dry weight ratio, and lung permeability index was markedly lower in the treatment group, and IL-6 and IFN-γ levels were significantly lower compared with the control group. The summary of the previous work on A1AT and IRI is shown in Table 11

The specific effect of A1AT in lung endothelium hasn’t been described yet. There is a study published that showed that A1AT facilitates acute response of the endothelium to TNFα in endothelial cell cultures. Another study showed that A1AT decreased apoptosis and reduced permeability, but it was also tested on cell cultures, this time human umbilical vein endothelial cell cultures. This studies are encouraging, however they fail to represent a more real-life ischemia reperfusion scenario.

Table 11 Summary of the previous studies and principal results on A1AT and IRI

<table>
<thead>
<tr>
<th>Model</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial Cell Culture</td>
<td>Cell morphology change, Caspase 3, IL-6 and IL-8</td>
</tr>
<tr>
<td>Rat IR</td>
<td>Lung oxygenation function, IL-1 alpha and TNF alfa</td>
</tr>
<tr>
<td>Pig Left Single Lung Tx (non survival)</td>
<td>Lung oxygenation, Wet-to-dry weight ratio and lung permeability, Neutrophil Elastase, IL-6 and interferon IFN-γ</td>
</tr>
<tr>
<td>Pig EVLP</td>
<td>Lung function, Pulmonary edema, IL-1α and IL-8</td>
</tr>
</tbody>
</table>

An IR epithelial cell culture model showed that A1AT prevented cell morphology change; A1AT reduced cell death via caspase-3 inhibition and reduced pro-inflammatory cytokine (IL-6 and IL-8); A1AT inhibited IR-induced loss of cell viability. In a rat IR model lung oxygenation function of rats that received A1AT was significantly better. A1AT treatment reduced the lung injury
score, neutrophil infiltration in the lung tissue and IR-induced lung epithelial apoptosis. A1AT reduced plasma levels of IL-1α and TNFα. On a pig left single transplant model with 4 hours of reperfusion A1AT significantly improved gas exchange, wet-to-dry weight ratio, and lung permeability index. IL-6 and IFN-γ levels were significantly lower compared with the control group.

The drug developing process must go through several stages in order to prove that a drug is safe, efficacious and that passes all the regulatory requirements, see figure 7. It first starts with target identification of the biochemical mechanism involved in a specific condition. The first phases include a preclinical research (basic science) on cells (in vitro studies) and small animals. The basic science work leads to understanding of how a potential drug works. In the complex field of lung transplantation, one of the major challenges can be the lack of translation of basic science discoveries to clinical settings. The existing FDA guidelines on new drug development require the demonstration of effect in more than one animal species, preferable a large animal. (Allio 2016) This is one of the reasons why, despite the positive findings regarding the use of A1AT to reduce IRI, there is still a need to test it in a big animal survival model and if results are positive, use human rejected lungs as a translational research model. Using a pig lung transplant survival model can potentially test the efficacy and safety of A1AT given to recipients, with specific emphasis on clinical outcomes, before moving to a clinical trial.

All the basic science studies (in cells and animals) done previously, have used cold ischemia time (more or less prolonged) as a model of injury. This type of model is simple and reproducible, however it is not representative of the lung transplant clinical scenario. Studying A1AT in clinically rejected human lungs enables us to test the drug in a very relevant and clinically representative scenario where we can address the complex and variable injuries that happen during to the donor lungs before and during the retrieval surgery and preservation.
The drug developing process must go through several stages in order to prove safety, and efficacy and to pass all the regulatory requirements. It starts with identification of the biochemical mechanism involved in a disorder. The first phases involve preclinical research (basic science) on cells (in vitro studies) and small animals. The latest guides suggest having the drug tested in another specie preferable a large animal model. Next step is to investigate if it can be studied in people in order to be able to translate the findings intoa clinical scenario. The specific effects are tested in efficacy studies and, if the results are encouraging, outcomes and effectiveness are tested to see how well the drug can work in a real-life setting.
During the last years we have tested A1AT in basic science scenario including cell culture (IR in epithelial cell culture), small animals (rat IR model), non-survival large animal model (pig single lung transplant), and during EVLP in pig lungs. Next steps are in red: 3-day survival pig lung transplant model and treatment during EVL to human rejected lungs.

2.2. Hypothesis

a. A1AT attenuates ischemia-reperfusion injury through anti-inflammatory and vasculoprotective properties

2.3. Objectives

a. To build a pig lung transplant survival model to test ischemia reperfusion injury.

b. To evaluate the safety and efficacy of A1AT in a three day porcine lung transplant survival model.

c. To evaluate the effect of A1AT during EVLP in human rejected lungs and its effect on lung vascular endothelium.
Chapter 3. Development of a Pig Lung Transplant Survival Model to Test Strategies to Reduce Ischemia Reperfusion Lung Injury

This Chapter has been modified from the following:

3.1. Abstract

Whilst lung transplant is a life-saving therapy for some patients, primary graft dysfunction (PGD) is a leading cause of mortality and morbidity soon after the transplant. Ischemia reperfusion injury is known to be one of the most important factors in PGD development. PGD is by definition an acute lung injury syndrome that occurs during the first three days following lung transplantation. In order to successfully translate laboratory discoveries to clinical practice, a reliable and practical large animal model is crucial. This three-day model enables the study of PGD.

This protocol describes the surgical technique and post-operative management required for swine lung transplantation and the subsequent three days of survival. Included in this protocol is the background and rationale, required supplies, and a detailed description of the donor operation, transplant surgery, post-operative care, and sacrifice surgery. This protocol reliably produces a pig lung transplant model in which the recipients survive for three-days post-transplant. The surgical procedures and necessary medical considerations are detailed in this protocol so that the three-day survival model can be used by lung transplant researchers. This model is useful for assessing the development of PGD, and for testing therapeutic strategies targeting PGD. In total, the protocol requires 300 minutes for the surgeries, plus approximately 120 minutes in total for the post-operative care.

3.2. Introduction

Lung transplantation is a life-saving therapy for end-stage pulmonary diseases. Animal models were the cornerstone for surgical development of the procedure prior to the first successful clinical lung transplant with long-term survival in 1983. (Hardy 1999; Demikhov 1969; Hardin and Kittle 1954; Cooper et al. 1987)
They have continued to be invaluable in pre-clinical research related not only to the surgical, but also to the immunological aspects of lung transplantation.

Lungs that are transplanted are often injured. Several factors can cause this, including the mechanism of death of the donor, the donor surgery, and the preservation necessary to transport the lungs. This injury can be further compounded by the transplant procedure itself as well as by the ischemia-reperfusion process and early immune responses following restoration of perfusion to the allograft after implantation.

Clinically, lung injury manifests itself as primary graft dysfunction (PGD), which is the most serious early complication after lung transplantation. The incidence of severe PGD is 11-25% and is associated with a 20-30% mortality rate in the first month after lung transplant (Christie et al. 1998; Christie et al. 2010; Christie, Carby, et al. 2005; Whitson et al. 2007). Along with the injury, there is significant innate immune activation in the lung allograft which is thought to lead to activation of donor-specific alloimmune responses, i.e. rejection. PGD has also been linked to increased rates of chronic lung allograft dysfunction. (Whitson et al. 2007; Christie, Kotloff, et al. 2005; Bharat et al. 2008)

Here, we present a three-day pig orthotopic single lung transplant model that we have used previously to study PGD and the early immune activation after lung transplantation (Pierre et al. 1998). The length of survival in this model can be extended for up to seven days (Machuca, Cypel, Bonato, Yeung, Chun, Juvet, Guan, et al. 2017), and even longer based on the goal of the study. In addition to assessing the consequences of ischemia reperfusion injury, this model has been used to assess the result of therapeutic treatments administered directly during surgery (Iskender et al. 2016; Martins et al. 2004), or during ex vivo lung perfusion (Machuca, Cypel, Bonato, Yeung, Chun, Juvet, Guan, et al. 2017; Yeung, Wagnet, et al. 2012). It can also be used to train new lung transplant surgeons and surgical teams.

Disadvantages of alternative animal models of lung transplantation
Canine models were critical in the initial development and breakthroughs in surgical technique and immunosuppressive regimens, which led to the first successful human lung transplantation (Karimi et al. 2011; Cooper et al. 1987). However, the use of canines has decreased significantly due to cost and ethical considerations. Lung transplants can also be performed in primates (Aoyama et al. 2015). These animals are prohibitively expensive for most research purposes.

Investigation of alloimmune mechanisms has been significantly advanced through the use of murine heterotopic, intra-pulmonary and orthotopic tracheal transplant models (Hertz et al. 1993). Murine lung transplant models provide a useful platform for more detailed investigation of early reperfusion injury and alloimmune responses to pulmonary grafts, and have opened access to the use of genetic and molecular tools that are necessary for mechanistic studies of alloimmune and non-alloimmune factors contributing to lung graft failure. During the 1990s the most commonly used rodent (mouse or rat) model to study lung rejection was the procedurally straightforward heterotopic tracheal transplantation, wherein the donor trachea is transplanted into the subcutaneous tissue of a recipient. A disadvantage of this model is the lack of information about parenchymal lung injury during acute rejection. The murine orthotopic lung transplant model is the closest representation of the acute rejection response seen in humans, as it utilizes a vascularized graft; however, this model is limited in that its technical requirements present a challenge to researchers and result in a low output. One way to increase output in mouse lung transplantation models is to use the intra-pulmonary tracheal transplant model of acute rejection. While this provides some information about rejection and lung injury in the pulmonary parenchymal milieu, it does not reflect the classic immediate ischemia-reperfusion injury of clinical lung transplantation which is a major contributor to early immune responses and PGD (Lama et al. 2017; Sato, Keshavjee, and Liu 2009).
The murine lung transplantation model can be attractive to researchers because of the reproducibility, reliability, and relatively low cost and personnel required. The ability to study specific factors in knock out animals and the availability of murine reagents also make the mouse transplant model attractive. However, all too often, promising therapies in murine models have not translated into clinical efficacy in humans; in fact, current FDA guidelines require testing of new pharmaceutical agents in both small and large animal models when proving a therapeutic concept. Thus, for numerous reasons, a large animal model of lung transplantation is important.

**Development of the Protocol**

Pigs and humans have anatomical and physiological similarities. Humans share far more immune-system-related genes and proteins with pigs than they do with mice or other animals. (Karimi et al. 2011) Our group, among others, were therefore interested in creating a sustainable, reproducible large animal model for lung transplantation. Anatomical similarities between pigs and humans means that general techniques for clinical lung transplantation can be applied to swine lung transplantation. However, one major anatomical difference is the right lung of the pig, which is divided into four lobes and includes a tracheal bronchus (Judge et al. 2014). Thus, to maintain a technique similar to that used in the clinic and to provide support to the pig after reperfusion, a left single lung transplant is preferable. This method was used in many models of non-survival swine lung transplant wherein reperfusion is assessed for a few hours before sacrificing the animal (Iskender et al. 2016; Yeung, Wagnetz, et al. 2012; Martins et al. 2004). We showed that pigs develop PGD that is similar in clinical and histopathological features to that seen in man after lung transplantation (Iskender et al. 2016). The gold standard in clinical practice is to assess patients for severity of PGD for 72-hours post-transplant. Using a three-day orthotopic single lung transplant model allows for the relevant 72-hour follow-up.
Changes seen in the transplanted lung over the first three days are most likely related to donor graft quality and early inflammatory injury. After this period, e.g. five to seven days, acquired immune responses start to play a bigger role. This model therefore enables a clinical assessment of recovery from the operation as the pigs are extubated after surgery. The choice of a single lung model also enables the researchers to regulate the desired injury level of the donor lung, as the native right lung supports the pig early on even if the donor lung exhibits severe PGD. The donor lung injury level can be altered in many ways. One example is by changing the duration of preservation, with prolonged cold preservation leading to more severe early lung injury after transplant. According to our experience, the clinical, functional, and histopathological changes seen in this model correlate closely to those seen in clinical lung transplantation.

The similarities between human and pig lung transplantation procedure, however, also present a limitation to the model, as performing a pig lung transplantation is complex and requires the experience of a trained thoracic surgeon to successfully complete. That being said, while a thoracic surgeon is required to take the lead in the surgeries, competent graduate students can assist with surgeries, perioperative and post-operative care. In addition, a survival model requires more complicated and demanding peri- and post-operative management. However, it has been shown to provide invaluable information about the effects of potential therapeutics (Madariaga et al. 2016; Pierre et al. 1998; Machuca, Cypel, Bonato, Yeung, Chun, Juvet, Guan, et al. 2017). When conducting a swine lung transplant survival model, it is imperative to take into account the fragility and size of swine lungs, which complicate the transplantation procedure.

3.3. Material and Methods

Experimental design
The major components of the Procedure are the donor surgery (Steps 1–38), recipient surgery (Steps 39–68), postoperative care (Steps 69–71), and sacrifice (Steps 72–99). The donor surgery is a bilateral pneumonectomy, which is performed using protective ventilation to avoid ventilator-related injury. The lungs are preserved in low-potassium dextran solution with prostaglandin E1 on ice until the time of implantation. The recipient surgery involves a left thoracotomy, left pneumonectomy, and lung implantation, which includes anastomosis of the bronchus, pulmonary artery (PA), and the left atrium. Postoperative care includes immunosuppression, antibiotics, and antithrombotic prophylaxis. Pain management is critical at this point to ensure comfort and survival of the recipients. The sacrifice surgery requires a sternotomy to clamp the right hilum in order to assess isolated graft function. This model can be extended beyond the 72-h survival described here. For instance, one study published by our group describes the application of this model to 7 d of survival; all pigs remained in good health at this point, suggesting that survival could be extended further based on the experimental design of the researcher.

**Level of expertise needed to implement the protocol**

The model is technically very demanding, and many lessons were learned during the development phase. Given the complexity of the lung transplant procedure, we recommend having at least one trained surgeon for the surgical procedure. All three surgeries (donor, recipient, and sacrifice) should be performed by at least two persons (a principal surgeon and one assistant). The postoperative management can be implemented by other lab members.

**Pig acclimatization**

Have pigs in the animal facility at least 1 week in advance to allow for acclimatization to the unfamiliar environment before the transplantation. The researcher should interact with the animals
in order to make them feel comfortable with people. Ideally, pigs should weigh between 25 and 30 kg, which will make the post-transplant management easier.

**Surgery**

Although pigs and humans have similar anatomies, there are four key surgically important anatomical differences between pigs and humans in terms of lung transplantation. First, the right upper lobe bronchus in the pig arises directly from the trachea, complicating right-lung transplant. Second, the left hemiazygos vein is fully developed and courses upward through the posterior mediastinum, where it crosses the left pulmonary hilum and drains directly into the coronary sinus. Third, the right inferior pulmonary vein crosses the midline to drain into the left atrium immediately adjacent to the left inferior pulmonary vein. Finally, the pig has a mediastinal lung lobe, which is anatomically part of the right lung.

**Administration of therapeutics**

Should your study involve assays of the effects of therapeutics, animals should be randomly assigned to different experimental groups (e.g., control versus treatment). It is recommended that the therapeutics to be tested should be given in a blinded manner throughout the protocol.

**MATERIALS**

**REAGENTS**

**CRITICAL** The reagents and surgical instruments below are those used in our laboratory. Similar equipment and reagents can be used as alternatives according to investigator preference and availability.

Donor and recipient animals: Yorkshire swine; domestic; males; 25-30 kg (approximately 3 months old). We have used Yorkshire swine successfully in our research. Swine lung transplant
models have been reported using other strains, including miniature pigs (Allan et al. 2002; Sato, Keshavjee, and Liu 2009).

**CRITICAL** There is a significant incidence of pneumonia in outbred pigs and generally it is good to have an extra pig for every transplant in case either the donor or recipient does not meet the selection criteria listed above. Do NOT use pigs if they have a PO$_2$ < 300 mmHg or any sign of infection as the intraoperative mortality in these pigs is very high.

**CAUTION** Any experiments involving pigs must conform to relevant Institutional and National regulations. We obtained approval from Animal Resource Centre, University Health Network (Toronto, Canada) to carry out the procedure described here. Animal care was in compliance with Principles of Laboratory Animal Care by the National Society for Medical Research and the Guide for the Care of Laboratory Animals by the National Institutes of Health.

Ketamine (40 mg/kg; Vetoquinol, Lavaltrie, QC, Canada; cat. no. 02374994)

Midazolam (0.3 mg/kg; Fresenius Kabi Canada Ltd., Richmond Hill, ON, Canada; cat. no. 02242905)

Atropine (Atro-SA® 0.04 mg/kg; Rafter 8, Calgary, AB, Canada cat. no. 00238481)

Fentanyl patch (2 x 25 µg/h; Sandoz, QC, Canada; cat. no. 02327120)

Inhaled isoflurane (3-5%; Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada; cat. no. 02231929)

PMS-Propofol (10 mg/mL; PharmaScience Inc., Montreal, QC, Canada; cat. no. 02244379)

Remifentanil Hydrochloride (1 mg/100mL PMS-Propofol; Teva Canada Limited, Toronto, Canada; cat. no. 023444424)

Methylprednisolone (1 g, Solu-mederol®; Pfizer; Kirkland, QC, Canada; cat. no. 02367963)

Heparin (300 U/kg (times 3); Leo®, Leo Pharma, Thornhill, Ontario, Canada; cat. no. 00453811)
Prostaglandin E\(_1\) (500 mg; Prostin\textsuperscript{®}, Pharmacia & Upjohn Inc., Mississauga, Ontario, Canada; cat. no. 00559253)

Low-potassium dextran glucose preservation solution (LPDG) solution (60 ml/kg; Perfadex\textsuperscript{®}; XVIVO Perfusion AB, Göteborg, Sweden; cat. no. 19001)

Calcium (1000 mg/10 mL of LPDG; Calciject, Omega, Montreal, QC, Canada; cat. no. 00821780)

Addex\textsuperscript{®}-THAM (0.3 ml/L of LPDG; Fresenius Kabi AB, Uppsala, Sweden; cat. no. 2000254-02)

Lignocaine (10 mg/0.1 mL); (3 pumps sprays of 10 mg) non-aerosol topical anesthetic; Astra Zeneca; cat. no. 39699/0086; Macclesfield, UK)

Lidocaine (100 mg/5 mL) (7 ml; Lidocaine HCl Injection USP, Alveda Pharmaceuticals Inc, Toronto, ON, Canada; cat. no. 02421992)

NaCl 0.9\% (9 g/L) (Saline solution (30 ml/h Baxter, Mississauga, ON, Canada; cat. no. JE1323)

Cefazolin (1 g; Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada; cat. no. 02237138)

Buprenorphine intramuscular (0.01 mg/kg; Vetersgesic\textsuperscript{®}; Champion Alstoe Animal Health, Whitby, ON, Canada; cat. no. 02342510)

Famotidine (10 mg/mL; Famotidine Omega, Omega, Montreal, QC, Canada; cat. no. 02247735)

Bupivacaine (0.5\% (5 mg/ml); Sensorcaine, Astra Zeneca, Mississauga, ON, Canada; cat. no. 02247735)

Heparin-Saline: 10,000 IU Heparin (Leo\textsuperscript{®}, Leo Pharma, Thornhill, Ontario, Canada; cat. no. 00453811) in 1L NaCl 0.9\% (9 g/L) Saline solution (Baxter, Mississauga, ON, Canada; cat. no. JE1323)

Ceftazidime (1 g/q12h; Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada; cat. no. 02304759)
Enoxaparin sodium (30 mg/q24h; Lovenox®, Sanofi-Aventis, Laval, QC, Canada; cat. no. 02236883)

Ciprofloxacin (2 mg/mL in 5% (50 g/L) Dextrose Solution; Sandoz, Boucherville, QC, Canada; cat. no. 02304759)

Cyclosporin (5 mg/kg; Novartis Pharma Canada Inc., Dorval, QC, Canada; cat. no. 02150697)

CAUTION The follow medications are restricted drugs and should be managed following the regulations: ketamine, midazolam, fentanyl patch, remifentanil, buprenorphine.

EQUIPMENT

Angiocath (Angiocath Autoguard Shielded IV Catheter, Becton Dickinson Infusion Therapy Systems Inc, Sandy, UT, USA; cat. no. 381701)

Cautery pad (Valleylab® Non-REM Polyhesive® Patient Return Electrode, Covidien, Mansfield, MA, USA; cat. no. E7506)

Cautery pencil (Valleylab® Button Switch Pencil, Covidien, Mansfield, MA, USA; cat. no. E2516)

Suction tip (Argyle® Yankauer, Covidien Mansfield, MA, USA; cat. no. 8888505123)

Suction tube (Argyle® Suction Tubing Molded Connectors Sterile, Covidien, Mansfield, MA, USA; cat. no. 43450)

IV Administration Set (Smiths Medical, St Paul, MN, USA; cat. no. 21-0442-25)

7.5 mm endotracheal tube (7.5 mm Sheridan/CF® Murphy Eye, Teleflex Medical, North Carolina, USA; cat. no. 5-10115)

6.5 mm endotracheal tube (6.5 mm Sheridan/CF® Murphy Eye, Teleflex Medical, North Carolina, USA; cat. no. 5-10115)

0-Silk ties (Perma-Hand Silk 0 (3.5 metric 30´´), Ethicon® Johnson and Johnson, Guaynabo, Puerto Rico; cat. no. A306)
Umbilical tape (Cotton Umbilical Tape, Ethicon®, Markham, ON, Canada; cat. no. U10T)

21 French PA Cannula (21 Fr Venous Catheter, Maquet, Hirrlingen, Germany; cat. no. 145-021)

4-0 Prolene (4-0 Surgipro™ II, Covidien, Mansfield, MA; cat. no. VP-581-X)

5-0 Prolene (5-0 Surgipro™ II, Covidien, Mansfield, MA; cat. no. VP-725-X)

0 Prolene (Ethicon, Peterborough, Canada; cat. no. 8424)

3-0 Vicryl (3-0 Polysorb™, Covidien, Mansfield, MA; cat. no. SL-5638)

2-0 Silk (2-0 Sofsilk™, Covidien, Mansfield, MA; cat. no. SS677)

0 Silk (0 Sofsilk™, Covidien, Mansfield, MA; cat. no. SS678)

Organ bags (Steri-Drape, 3M Health Care, St. Paul, MN; cat. no. 1003)

IV Catheter (Cook TPN® Single Lumen Catheter Set, Cook Group, Bloomington, IN; cat. no. G08132)

Feeding Tube (8Fr Feeding Tube, Covidien, Mansfield, MA; cat. no. 8888260406)

Perfusion system (IV Administration Set, Smiths Medical ASD Inc., St. Paul, MN, USA; cat. no. 21-0442-25)

Tourniquet (TourniKwik 6 in (15.2 cm), Medtronic, Minneapolis, MN, USA; cat. no. 79011)

11 Blade (Feather Surgical Blade Stainless Steel, Feather Safety Razor Co. LTD., Osaka, Japan; cat. no. 504170)

3 ml Syringe (BD 3 ml Syringe Luer-Lok™ Tip, Franklin Lakes, NJ, USA; cat. no. 309657)

5 ml Syringe (BD 5 ml Syringe Luer-Lok™ Tip, Franklin Lakes, NJ, USA; cat. no. 309646)

18 Gauge needle (BD SafetyGlide™ 18 Gauge Needle, Franklin Lakes, NJ, USA; cat. no. 305918)

25 Gauge needle (BD SafetyGlide™ 25 Gauge Needle, Franklin Lakes, NJ, USA; cat. no. 305901)

24 French Chest tube (Argyle™ Thoracic PVC Catheter 21Fr/Ch, Covidien, Mansfield, MA; cat. no. 8888570531)
Heimlich valve system (Pneumostat™, Atrium, Hudson, NH; cat. no. 16100)

3-Way Stopcock (Discofix 3-Way Stopcock blue, B. Braun Medical Inc., Bethlehem, PA, USA; cat. no. D300)

Ventilator (SERVO-i® Ventilator, Maquet Critical Care AB, Solna, Sweden; cat. no. 06449701)

Adult flexible fiber-optic bronchoscope (Olympus EVIS EXERA II BF type Q180, Japan)

Bronchoscope tower (Olympus Exera II CLV-180, Japan)

Single Use Suction Valve (Olympus, Japan; cat. no. MAJ-209)

Pressure monitor (PM-9000Vet; Mindray Medical, Shenzen, China; cat. no. V9204)

Blood gas analyzer (RAPIDpoint® 500, RAPIDLab Systems, Siemens, Erlanger, Germany; cat. no. 115823)

Isoflurane vaporizer (Moduflex Optimax, Dispomed Ltd., Joliette, QC, Canada; cat. no. 5179)

4°C fridge (Foster Refrigerator of Canada, Drummondville, QC, Canada) with temperature-recording device (Honeywell, Minneapolis, MN)

WarmTouch Patient Warming System (Tyco Healthcare Group, Nellcor Puritan Bennett Division; Pleasanton, CA, USA; cat. no. 5015300A)

Heating Pad (T/Pump Localized Therapy System, Stryker, Hamilton, ON, Canada; cat. no. TP700)

Sternal saw (Stryker Autopsy Saw #810, Stryker; cat. no. BD001)

PROCEDURE

CAUTION All experiments involving animals must be performed in accordance with national and institutional regulations.

Preparation of recipient. TIMING: 10 min.

1. If you will be proceeding to recipient surgery within the 24 h period after the donor operation, apply a 50 μg fentanyl patch 24 h before the start of recipient surgery (step 39)
to a shaved area on the pig’s back. Make sure the animal cannot reach it. Cover the patch with plastic to protect and secure it.

**CRITICAL STEP** Postoperative pain management is an important step in the survival model. The fentanyl patch ensures analgesia from the beginning of the surgery and throughout the postoperative period.

**Donor operation. TIMING: 40-50 min.**

**CRITICAL STEP** Donor lung quality is essential for the reproducibility of the experiments.

2. Ensure pigs are delivered to the research facility a minimum of 3 days prior to surgery to acclimatize to the facility and reduce stress.

3. Sedate the donor pig with ketamine (20 mg/kg, IM), midazolam (0.3 mg/kg, IM) and atropine (0.04 mg/kg, IV) and wait until the pig is completely calm. This sedation can be given in the holding pen prior to transfer to the operating room.

4. In the operating room, start inhaled isoflurane (3-5%) with 2-3 L/min oxygen via face mask.

5. Leave the pig in prone position while putting an IV ear line with a 22 g angiocath (Figure 9a).

**CRITICAL STEP** Reduce the time the pig stays on his back as much as possible to avoid atelectasis.

6. Administer cefazolin (20 mg/kg, IV) and methylprednisolone (500 mg, IV).

7. Connect the propofol (100 mL) containing remifentanil (1 mg) to the IV line without starting the infusion. Have all the instruments to perform a tracheostomy ready on a side table.

8. Turn the pig with the upper legs positioned above the head.

9. Give a 7-10 ml (70-100 mg) bolus of propofol before tracheotomy.
10. Make a 10 cm vertical midline neck incision with cautery. Dissect between the strap muscles until you reach the trachea. Go around the trachea and place a 0-silk tie. Use the tie to retract the trachea and make a 1 cm incision between 2nd and 3rd tracheal rings. Insert a 7.5 French endotracheal tube, making sure the balloon remains partially visible to avoid advancing the tube too distally. Inflate the balloon (Figure 9b).

11. Start propofol (5-8 mg/kg/h, IV), and remifentanil (2-20 µg/kg/h, IV).

12. Place the pig on pressure-controlled (PC) ventilation at 15 cmH₂O, PEEP of 5 cmH₂O, FiO₂ of 0.5 and respiratory rate of 15/min targeting tidal volumes of 6-8 ml/kg, normal pO₂, and normal pCO₂ levels. The pressure and respiratory rate should be adjusted accordingly to remain in the target range specified above.

13. Clean the skin with betadine.


15. Open the peritoneum, take the abdominal organs (bowels and stomach) out and put them in a green towel to relieve pressure on the diaphragm.

16. Dissect with the finger under the sternum (from above and below) separating the pericardium from the sternum. Divide the xiphoid and place a Kocher clamp on each side to pull it down.

17. Perform a median sternotomy and achieve hemostasis on the bone edges.

18. Inject sodium heparin (10,000 U, IV).

19. Encircle the inferior vena cava above the diaphragm with a silk tie, and identify and divide the pulmonary ligaments on each side with the cautery to free the lung.

**CRITICAL STEP** Liberating the pulmonary ligaments will allow better recruitment of the lung before lung flush which is vital for the 24h hypothermic preservation model.

20. Excise the thymus gland and open the pericardium.
**CRITICAL STEP** After opening the pericardium, ensure hemodynamic stability of the pig before continuing to the next step.

21. Dissect the superior vena cava, moving the heart carefully to one side and then the other, to see where the SVC ends. Encircle the cava with a silk tie.

22. Divide the space between the pulmonary artery (PA) and the aorta.

23. Use a 4-0 prolene suture to put a purse-string with a tourniquet in the mid portion of the PA (about 2 cm distal to the valve) (*Figure 9c*). Incise the PA in the center of your purse-string suture using an 11 blade. Gently use digital pressure to prevent excessive bleeding. Gently expand the hole in the PA using a hemostat clamp. Insert a 21 French cannula into the PA, and then clamp the cannula (*Figure 9d*).

**CRITICAL STEP** Make sure the tip of the cannula is positioned *proximal* to the PA bifurcation to ensure uniform flushing of both lungs. Place a silk tie around the cannula and tourniquet to secure it.

24. Connect the perfusion line to the PA cannula, keeping it clamped at this time.

25. Take arterial blood from the aorta and venous blood from the inferior vena cava for blood gas analyses. The arterial pO$_2$ should be >300 mmHg.

**CRITICAL STEP** If the pO$_2$ is <300 mmHg, the donor should not be used for transplantation.

26. To minimize pulmonary atelectasis, do a volume recruitment maneuver with a sustained airway pressure of 25 cmH$_2$O for 3 seconds, repeated every 30 seconds for a total of 3 times. Avoid recruitment duration longer than 3 seconds as prolonged recruitment causes hypotension, and leads to lung injury in the pig.
27. Prepare lung preservation solution by adding 0.6 ml/L Tham and 0.3 ml/L of calcium to Perfadex solution (XVIVO Perfusion, Denver, CO), following the instructions. A total of 3 L of Perfadex is prepared as outlined above. Inject 500 µg of Prostaglandin E1 (PGE₁) into the 3 L of Perfadex solution.

28. After the lung preservation solutions are prepared, inject 1 ml (500 µg) of PGE₁ directly into the PA and wait 15 seconds to allow for sufficient vasodilation, as evidenced by a drop in blood pressure.

29. Clamp the aorta (Figure 9e).

30. Ligate the superior and inferior vena cava.

CRITICAL STEP Transect the left atrial appendage (creating a 2 cm diameter hole for the effluent flush solution to drain into the pleural space for topical cooling of the lung) and flush the PA with 60 ml/kg of Perfadex solution at 4°C from a height of 30 cm above the heart.

31. Pull the diaphragm down to allow lung expansion.

32. After the antegrade flush, gently lift the left lung over to the right side. At this stage, it is critical to avoid excess manipulation of the lung (Figure 9f). TROUBLESHOOTING

CRITICAL STEP: In the pig, any contact with the lung that is not gentle can result in mechanical trauma, which manifests as blebs on the pleural surface.

33. Use the esophagus as reference and dissect anterior to it, starting inferiorly and extending all the way up to the aortic arch (Figure 9g). Then transect the aorta. Do the same on the other side. Dissect in the neck to expose the trachea. Identify the space between the trachea and both lung apices, and dissect everything in between them.

34. Continue ventilation throughout the lung extraction procedure.
35. Excise the heart, making sure to preserve the main PA just proximal to the bifurcation and as much atrial cuff as possible.

36. Using a foley catheter with the balloon inflated, perform a retrograde flush from the left atrium through each pulmonary vein with 15 ml/kg of Perfadex (a quarter of the volume instilled into each vein) in situ, while the lungs are still being ventilated (Figure 9h).

37. Clamp the trachea with 2 clamps, keeping the lungs inflated with a sustained airway pressure of 15 cmH$_2$O. *Do not hyper-inflate the lungs (Figure 9i).* Remove the double lung block and place it in the pre-prepared organ bag containing 1L of cold Perfadex which is placed on ice to keep cool.

38. Use gauze sponges (wet with Perfadex solution) to protect the lung surface and specifically ensure the clamps do not damage the lung. Place the lung block in a plastic bag (double bagged) containing 1 L of 4°C Perfadex solution.

**PAUSE POINT** The lung can be stored at 4°C in a refrigerator or in a cold room. In our experience, cold storage of up to 30 h has been successfully used. (Pierre et al. 1998)
This study was conducted with the approval from the animal care committee of the Toronto General Hospital Research Institute. All animals received humane care in compliance with the guidelines of the Toronto General Hospital Research Institute. Animals were used in accordance with the recommendations of the Toronto General Hospital Animal Care Committee.
“Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

Recipient operation. TIMING: 180-200 minutes

CRITICAL STEP A 50 µg fentanyl patch must have been applied 24 h before the recipient surgery to a shaved area on the pig’s back as detailed in step 1.

39. Sedate the pig as described in the donor operation steps 3-6 above. Connect the propofol (100 mL) containing remifentanil (1 mg) to the IV line. Give a 7-10 ml (70-100 mg) bolus of propofol, but do not start the continuous infusion.

40. Ensure the pig is in a supine position. One person should hold the mouth open with the tongue out and away from the mouth. Apply lubricant to the 6.5 mm endotracheal tube. Use the laryngoscope to depress the tongue and pass through the oral cavity. Use the tip of the endotracheal tube to move the epiglottis out of the way of the laryngeal opening. Spray lidocaine solution to the vocal cords, remove the laryngoscope, close the mouth, and wait for two minutes for the lidocaine to act. Open the mouth and replace the laryngoscope as before, and advance the endotracheal tube into the trachea, rotating slightly. Secure the endotracheal tube in place by inflating the cuff. Ensure the tube is properly placed by performing thoracic auscultation. Tape the snout of the pig closed to ensure the tube does not move throughout the procedure. Begin a continuous IV infusion of propofol (5-8 mg/kg/h, IV) and remifentanil (2-20 µg/kg/h, IV).

41. Administer eye lubricant (artificial tears) to each eye to protect from corneal ulcers. Use a heating pad under the pig and a heating blanket to maintain body temperature.

CRITICAL STEP Administration of eye lubricant needs to be repeated every 2-3 h.
42. Ensure cefazolin (20 mg/kg, IV) and methylprednisolone (1 g, IV) are administered 30 min prior to skin incision. Use NaCl 0.9% for volume replacement at a rate of 70-100 ml/h.

**CRITICAL STEP** Manage fluid infusion carefully. Do not exceed these volumes. If experimental drugs are administered, the saline volume should be reduced accordingly to avoid volume overload that can exacerbate reperfusion injury of the lung.

43. Move the left forelimb of the pig down and away from the head ensuring it is complexly extended, in order to maximize exposure to the neck. Make a 6 cm neck incision, 2 cm left of the mid line. Dissect parallel to the trachea until the jugular vein and the carotid artery are reached, and encircle with two 2-0 silk ties each. Place a clamp on the proximal artery and ligate the distal end. Open the artery and insert an 18 Fr catheter into the artery (insert about 10 cm into the carotid artery) and secure it with a silk tie. Do the same with the jugular vein. Ligate the distal end and place the IV catheter proximally. Attach the arterial line to a multichannel recorder to continuously monitor the blood pressure, and use the venous line to administer medications. These lines will stay in place for the postoperative care.

**CRITICAL STEP** If the pO2 is below 300 mmHg, the recipient should not be used for the experiment as there is high mortality in these animals. There are seasonal periods where pigs are susceptible to viral and bacterial pneumonias. If there is evidence of an infection, this of course will compromise the experimental results.

44. Turn the pig to a right lateral position to perform a 15 cm left thoracotomy. Use the scapula to find the 4\textsuperscript{th} intercostal space, open the pleural space and feel the costochondral rib junction with the sternum, and divide it (*Figure 10a*). Enlarge the thoracotomy if necessary.
Be careful while opening the posterior part of the intercostal space to avoid bleeding (Figure 10b).

**CRITICAL STEP:** Dividing the rib junction permits better access to the hilar structures and facilitates the anastomosis. Opening the posterior part of the intercostal space excessively can result in difficult to control bleeding.

45. Dissect the left azygos vein, elevate it from the left atrium and ligate it. Leave the proximal tie long so it can be used for retraction. Separate the lymph node from the PA and take it out to visualize the whole left PA. **TROUBLESHOOTING**

46. Encircle the vagus nerve to avoid injury (Figure 10c). Dissect the left PA proximally until the right PA is visualized. Make sure to not dissect excessively, and leave the PA adventitia intact to avoid rupturing the PA. Encircle the left main PA with umbilical tape (Figure 10d). Avoid injuring the phrenic nerve.

**CRITICAL STEP** The pig PA is much more prone to rupture than a human PA. Therefore, avoid dissecting the PA too much and leave the adventitia intact.

47. Dissect the pulmonary veins (superior and inferior). Reduce the pressure support on the ventilator to 8 cmH₂O temporarily, to reach for the inferior ligament and liberate it. After liberating the inferior ligament, return the ventilator settings to the level prior to this step (Figure 10e).

48. Administer a bolus of heparin (3,000 U, IV).

49. Prepare the donor left lung on the back-table. Ligate on the atrial side and cut distally both the mediastinal lobe vein and the right pulmonary vein – this preserves a large LA cuff (Figure 10f). Dissect the main PA and divide the left side from the right side. Trim the left PA, leaving about 1.5 cm in length on the graft to avoid kinking (Figure 10g). Make sure
to dissect to the first branch in order to identify it. Trim the atrium, leaving the 2 left pulmonary veins with a common cuff. Make sure to leave enough muscle to suture (Figure 10h.) Dissect the trachea to the carina. Open the bronchus and trim it to 2 rings proximal to the upper and lower lobe bifurcation.

50. Gently carry the left lung to the recipient table. Place it in the chest on a cold wet gauze.

51. Dissect the left main bronchus and place a clamp as proximally as possible. Place a vascular clamp proximally on the left main PA. Ligate and divide the left pulmonary veins (Figure 10i). Divide the PA as distally as possible. Divide the bronchus as distally as possible (Figure 10j).

**CRITICAL STEP** Reduce the tidal volume to 5–8 ml/kg and adjust the respiratory rate to keep the pCO₂ between 35–40 mmHg and the pO₂ over 95 mmHg. Hypercapnia from insufficient ventilation leads to a high risk of arrhythmia during subsequent atrial clamping.

52. Perform the bronchial anastomosis first. Put one suture in the top corner and do a running 4-0 prolene suture on the posterior and anterior walls (Figure 10k.). If you are performing experiments for extended survival, then an interrupted suture technique is preferred to avoid the development of anastomotic stenosis.

**TROUBLESHOOTING**

53. Trim the recipient pulmonary artery (usually around 1.5 cm) in order to avoid kinking (Figure 10l). Perform the PA anastomosis next with a continuous 5-0 prolene suture interrupted in two places and fill the lumen with saline injected through an angiocath to de-air it prior to tying the suture (Figure 10m). Before advancing to the next step, check that there is no obvious leaking from the PA anastomosis. **TROUBLESHOOTING**
**CRITICAL STEP:** Perform the atrial anastomosis with a continuous **everting horizontal mattress suture** using 5-0 prolene interrupted in two places. This everting horizontal mattress suture technique is essential to prevent the development of atrial anastomotic thrombosis.

54. To perform the atrial anastomosis, sew the donor and recipient atrial cuffs in a straight line and leave them apart from each other until the back wall stitches are all placed (*Figure 10n*). Pull snugly on the two ends of the suture to bring the anastomosis together (*Figure 10o*). Tie the second holding suture and then tie the first running suture to the holding suture. Sew the anterior wall halfway with the second suture, using an everting mattress suture technique again (*Figure 10p*). Use the remaining end of the first suture, which was initially left untied for the other half of the anterior wall. Keep the anterior wall suture untied at this point for later de-airing.

55. Give a bolus of heparin (3,000 U, IV) and wait for 5 minutes.

**CRITICAL STEP:** Repeating the heparin bolus is important to avoid thrombus formation.

56. Perform a bronchoscopy and open the bronchial clamp under direct vision. Re-inflate the transplanted lung, after suctioning all secretions inside the bronchus.

57. After re-inflation of the transplanted lung to a pressure of 20 cmH₂O, open the PA clamp partially (50%) and de-air the lung through the left atrial anastomosis. Once the lung is de-aired through the open atrial anastomosis, remove the atrial clamp and tie the two sutures together (*Figure 10q*). **TROUBLESHOOTING**

**CRITICAL STEP:** It is important to pull the atrial suture snugly before tying, to securely oppose the tissues and avoid bleeding through the everting mattress suture line.
**CRITICAL STEP:** Keep the PA clamp partially occluded and gradually release over 10 minutes 
to provide controlled initial reperfusion.

58. Ventilate the lungs using pressure control (PC) ventilation of 15 cmH$_2$O with 50-60% 
oxxygen, 5 cmH$_2$O PEEP, and respiratory rate adjusted to keep the pCO$_2$ between 35 and 
40 mmHg for 10 minutes, and take the first blood gas.

59. 10 minutes after reperfusion (step 58), start reducing FiO$_2$ from 0.6 to 0.5 depending on 
the blood gases.

60. Insert a 24 Fr (8 mm) chest tube hooked up to Heimlich valve system entering through the 
6$^{th}$ intercostal space (2 spaces below the thoracotomy).

61. Perform an intercostal block using a mixture of 5 ml bupivacaine (0.5%) and 5 ml xylocaine 
(1%) placed from 2 intercostal spaces above to 2 intercostal spaces below the 
thoracotomy for post-operative analgesia.

62. Evaluate the graft function after 1 hour of reperfusion by taking blood samples from each 
pulmonary vein for transplanted lung specific blood gases.

63. After 1 hour of perfusion also perform a recruitment maneuver with a sustained airway 
pressure of 25 cmH$_2$O for 3 s, repeated every 30 s for a total of 3 times. Avoid recruitment 
duration longer than 3 s as prolonged recruitment could induce hypotension at this stage.

64. After 1 h of reperfusion also perform a bronchoscopy to check that there is no edema fluid 
coming from the transplanted lung. If the bronchoscopy is clear and the blood gases are 
good, there is no need to perform another bronchoscopy.

65. If, 1 h after reperfusion, the bronchoscopy is clear and blood gases are good, close the 
chest in three layers: ribs (interrupted “figure of 8” 0 vicryl), muscle (running 2-0 vicryl), 
skin (running 3-0 silk). Close the neck incision and secure the central lines to the pig’s 
skin all the way to the upper back. Leave the jugular venous line for administration of 
medications postoperatively. If the bronchoscopy shows lung edema or if blood gases are
poor, consider leaving the pig sedated for one more hour before closing the chest. Two hours after reperfusion, if the blood gases are poor (PaO$_2$/FiO$_2$ ratio <200 mmHg), consider sacrificing the animal at this point.

66. If the blood gases after 1 hour are adequate, start weaning anesthesia by reducing propofol and fentanyl infusions by half every 15 minutes. Leave the arterial line for arterial blood-gas analysis.

67. Turn the pig into the prone position after closing the chest. Give the pig 3 ml buprenorphine (IM) analgesic.

68. When the pig starts breathing spontaneously, approximately 30 min after beginning wean of anesthesia, change the ventilator to pressure support mode and reduce ventilator support slowly. Leave the endotracheal tube in as long as the pig tolerates it. (Figure 11a) Extubate when the animal starts coughing or fighting with the tube. There should be an oxygen mask with 3-5L/min O$_2$ available to use as long as the pig remains immobile. In an uncomplicated operation, the pig is usually ready for extubation 2-3 h after reperfusion. After extubation, the pig can be returned to the holding pen and portable oxygen should be available in the holding area to deliver to the animal via face mask if necessary.
Pig in a right lateral position to perform the left thoracotomy (a). 15 cm thoracotomy in 4th intercostal space, costochondral rib junction with the sternum divided the posterior part carefully opened (b). Vagus nerve encircled (c). Left main PA encircled with an umbilical tape (d). Pulmonary veins dissected (e). Donor left lung on the back table: mediastinal lobe vein and right pulmonary vein ligated on the atrial side and cut distally (f). Donor PA trimmed leaving 1.5 cm in length on the graft (g). Donor atrium trimmed, the 2 left pulmonary veins have a common cuff (h). Recipient left pulmonary veins ligated and divided (i). Bronchus divided as distally as possible (j). Bronchial anastomosis with a running 4-0 prolene suture (k). Recipient bronchus anastomosed...
and PA trimmed (around 1.5 cm) (l). PA anastomosis performed with a continuous 5-0 prolene suture interrupted in two places being filled with saline injected through an angiocath (m). Atrial anastomosis with corner 4-0 prolene corner stitch (n). Back wall of the atrial anastomosis with an evertting mattress suture after pulling on the two ends of the suture to bring it together (o). Atrial anastomosis after the anterior wall was sewed using an evertting mattress suture technique (p). Reperfusion: PA clamp partially released atrial clamp released and the two atrial sutures tied together (q).

This study was conducted with the approval from the animal care committee of the Toronto General Research Institute. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

Postoperative care. TIMING: 120 min to over 60 h

69. Immediately postoperatively, monitor the respiration rate and pattern, heart rate, and EKG. (Figure 11b)

70. Treat signs of pain, such as vocalization, unwillingness to move, and tachycardia with buprenorphine (0.01-0.05 mg/kg IV q6h). Use standing buprenorphine orders (0.01-0.05 mg/kg IM q12) to relieve pain. Generally, the pigs start attempting to stand up 1 hour after extubation. (Figure 11c)

71. Over the remaining time post extubation, follow the drug regimen stated in Table 1. Before and after administering each medication, flush the line with normal saline with heparin to maintain patency. (Figure 11d)
Recipient pig in prone position waking up from anesthesia (a). Recipient pig in the recovery pen with an ear sensor to monitor pO₂ (b). Recipient pig being monitored with a tongue pO₂ pulse-oxymeter (c). Recipient pig in the post-operative pen receiving IV medication. IV lines and chest tube are wrapped around the chest to avoid being caught or chewed by the pig (d).
This study was conducted with the approval from the animal care committee of the Toronto General Research Institute. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

**Table 12 Post-operative drug regime**

<table>
<thead>
<tr>
<th>FREQUENCY</th>
<th>TREATMENT</th>
<th>DOSE</th>
<th>REASON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twice daily</td>
<td>Famotidine</td>
<td>20 mg/dose, IV drip</td>
<td>Ulcer prevention</td>
</tr>
<tr>
<td>Twice daily</td>
<td>Methylprednisolone</td>
<td>1 mg/kg/dose, IV drip</td>
<td>Immunosuppression</td>
</tr>
<tr>
<td>Twice daily</td>
<td>Cyclosporine</td>
<td>10 mg/kg/dose po</td>
<td>Immunosuppression</td>
</tr>
<tr>
<td>5 h post surgery</td>
<td>Enoxaparin sodium</td>
<td>30 mg/dose, SC</td>
<td>Prophylaxis of thromboembolic events.</td>
</tr>
<tr>
<td>12 h post surgery</td>
<td>Enoxaparin sodium</td>
<td>30 mg/dose, SC</td>
<td>Prophylaxis of thromboembolic events.</td>
</tr>
<tr>
<td>24, 48 and 72 h post surgery</td>
<td>Enoxaparin sodium</td>
<td>30 mg/dose, SC</td>
<td>Prophylaxis of thromboembolic events.</td>
</tr>
<tr>
<td>Twice daily</td>
<td>Ciprofloxacin</td>
<td>400 mg/200 ml/dose, IV drip</td>
<td>Antimicrobial prophylaxis</td>
</tr>
<tr>
<td>Twice daily</td>
<td>Ceftazidime</td>
<td>1 g/10 ml/dose, IV drip</td>
<td>Antimicrobial prophylaxis</td>
</tr>
<tr>
<td>End of surgery</td>
<td>Buprenorphine</td>
<td>0.01-0.05 mg/kg/dose, IV</td>
<td>Pain management</td>
</tr>
<tr>
<td>Twice daily</td>
<td>Buprenorphine</td>
<td>0.01-0.05 mg/kg/dose, IV</td>
<td>Pain management</td>
</tr>
<tr>
<td>Additional q6h as needed</td>
<td>Buprenorphine</td>
<td>0.01-0.05 mg/kg/dose, IV</td>
<td>Pain management</td>
</tr>
</tbody>
</table>
Third day measurements, sacrifice and biopsies. TIMING: 60 min

72. Sedate the pig using the same technique described in the donor operation above (steps 3-7).

73. Perform the tracheostomy as described in the donor operation (steps 8-11).

74. Start propofol (5-8 mg/kg/h, IV), and fentanyl citrate (2-20 µg/kg/h, IV).

75. Place the pig on a pressure-controlled ventilator at pressure control of 15 cmH₂O, PEEP of 5 cmH₂O, FiO₂ of 100% and respiratory rate of 15.

76. Perform a bronchoscopy, take a close look at the anastomosis to ensure there are no signs of airway complications (for instance stenosis, dehiscence, infection or necrosis) which could confound the results of the study. Suction the airways. Do not take research samples at this point.

77. Do a chest X-ray after the bronchoscopy. In some cases, mild bronchial plugging can cause lobar atelectasis that is completely resolved by suctioning the secretions during the bronchoscopy.

78. Make a midline incision (thoraco-abdominal).

79. Open the peritoneum, take the abdominal content (bowels and stomach) out and put them in a towel to relieve pressure.

80. Dissect the mediastinal tissue with the finger under the sternum (both sides), separating the pericardium from the sternum. Divide the xiphoid and place a Kocher clamp on each side to pull it down.

81. Perform a median sternotomy and achieve hemostasis of the bone edges with electrocautery.

82. Release the right pulmonary ligaments from the diaphragm.

83. Perform a volume recruitment maneuver with a sustained airway pressure of 20 cmH₂O for 3 seconds, repeating three times every 30 seconds. TROUBLESHOOTING.
84. Remove the thymus and open the pericardium.

85. Take an arterial systemic blood gas sample.

86. Pull the left side pericardium in order to localize the pulmonary veins clearly.

87. Take blood gas samples from each vein of the transplanted lung.

88. Clamp the right hilum with 2 clamps, one coming from the top and the other from the bottom. Make sure the tidal volumes are decreased due to clamping of the right lung, and specifically ensure that the separated mediastinal segment is not ventilating. A small clamp may be needed to clamp the mediastinal lobe. TROUBLESHOOTING

89. Wait for 10 minutes. During the right hilar clamping, the pigs may need hemodynamic support. If the systolic blood pressure drops below 70 mmHg, dopamine (5-15 μg/kg/min) can be given to support the pig while waiting for recovery.

90. Take an arterial blood gas to determine isolated graft pulmonary function.

91. Measure the compliance (static and dynamic).

92. Put an angiocath into the pulmonary artery and connect it to the pressure line to measure the PA pressure.

93. Unclamp the right hilum and let the pig recover.

94. Perform a bronchoscopy and bronchial washes or bronchoalveolar lavage for research samples.

95. Ligate the inferior vena cava and cut it to exsanguinate the pig.

96. Start extracting the lung by holding the lung carefully to the surgeon’s side. Using the esophagus as reference, dissect anterior to it starting inferiorly and extending all the way up until reaching the aorta. Cut the aorta. Do the same on the other side. Dissect the neck until you reach the trachea and find the space between the trachea and each side lung apex, and cut everything between them.
97. Continue ventilation throughout the extraction of the heart–lung block. Clamp the trachea with the lungs inflated with a sustained airway pressure of 15 cmH₂O. Take out the heart-lung block.

98. Open the PA to examine the arterial anastomosis and open the branches to see if there are any thrombi. Do the same with the atrium. Open the bronchus and examine the anastomosis.

99. Take all needed tissue biopsies.

**TROUBLESHOOTING**

See Table 13 for troubleshooting guidance.

*Table 13 Troubleshooting*

<table>
<thead>
<tr>
<th>STEP</th>
<th>PROBLEM</th>
<th>REASON</th>
<th>SOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>Flush comes out bloody from the atrium</td>
<td>Aortic clamp is not well positioned</td>
<td>Adjust the aortic clamp and make sure is clamping the whole vessel without clamping the PA</td>
</tr>
<tr>
<td>45</td>
<td>Poor exposure of the hilum</td>
<td>Small incision Chest entered in the wrong intercostal space</td>
<td>Enlarge incision Excise the upper or lower rib</td>
</tr>
<tr>
<td>52</td>
<td>PA torsion</td>
<td>First branch is not well positioned PA is too long</td>
<td>Make sure that the PA is in the right position Shorten the PA donor and/or recipient</td>
</tr>
<tr>
<td>52</td>
<td>PA bleeding</td>
<td>Stitches are not close enough</td>
<td>Clamp if needed and put an extra stitch</td>
</tr>
<tr>
<td>56</td>
<td>Atrial bleeding</td>
<td>Stitches are not close enough Stich is loose</td>
<td>Clamp if needed and put an extra stitch Pull the suture from both sides to oppose the tissues</td>
</tr>
<tr>
<td>82</td>
<td>Lung looks atelectatic</td>
<td>Bronchial obstruction</td>
<td>Suction all secretions with the bronchoscope</td>
</tr>
<tr>
<td>87</td>
<td>Native lung is ventilating after clamping the hilum</td>
<td>Clamp is not well positioned Clamp is not reaching the mediastinal bronchus</td>
<td>Adjust both clamps (top and bottom) Use a separate clamp to clamp the mediastinal lobe.</td>
</tr>
</tbody>
</table>
TIMING

Step 1-38, Donor operation: 40-50 minutes

Step 39-68, Recipient operation: 180-200 minutes

Step 69-71, Postoperative care: 120 minutes over 60 hours

3.4. Results and Discussion

This model aims to mimic the clinical setting as the pig and man have anatomical and physiological similarities, and the transplants are performed over a major histocompatibility class antigen mismatch. Apart from being surgically demanding, the model also requires medical and supportive therapy reminiscent of the perioperative period after clinical lung transplantation. During our model development, we had high early mortality related to both surgical and medical complications and we plan to not only describe the surgical procedures (donor surgery, recipient surgery, assessment, sacrifice) and medical management (pre- and postoperative care) in detail, but also go through the pitfalls we encountered during the development phase of this model.
Chapter 4. Preclinical Large Animal Survival Study of Alpha-1-Antitrypsin Treatment of Donor Lungs to Test Safety, Tolerability and Clinical Benefit

This Chapter has been modified from the following:


4.1. Abstract

**Background:** Ischemia reperfusion injury (IRI) has shown to have an important impact on early morbidity and mortality as well as late graft function. IRI activates innate immune responses and promotes a pro-inflammatory microenvironment that predisposes the lung to the development of PGD. Alpha 1 antitrypsin (A1AT) is a serine-protease inhibitor that inhibits neutrophil elastase. We among others have recently shown that A1AT has anti-inflammatory and anti-apoptotic properties in IRI. The aim of this pre-clinical study was to test the clinical effect as well as the safety and tolerability of supra-therapeutic doses of A1AT administered to recipients prior to reperfusion in a pig lung transplant 3-day survival model.

**Methods:** Donor lungs were stored for 24 hours at 4°C, followed by left single lung transplantation. Animals were randomized to either receive human A1AT (500 mg/kg) or saline before reperfusion, as well as at 24h and 48h post-reperfusion (n=6/group). Clinical outcome was measured by time to weaning of supplemental oxygen after transplant. Systemic toxicity was assessed by examining hematological, renal and hepatic function.

**Results:** Pigs in the treatment arm were weaned off oxygen significantly faster (22.5 min vs 125 min, p<0.002). There were no signs of hematological, renal, or hepatic toxicity observed in the A1AT treated group or the control group.

**Conclusion:** In this study, we show that A1AT is safe, well tolerated, and clinically beneficial in the setting of lung transplantation and may be able to help reduce IRI after transplantation.

**Keywords** pig survival lung transplant model, primary graft dysfunction, alpha-1-antritrypsin
4.2. Introduction

During lung transplantation, several factors influence early lung injury, including donor mechanism of death, preservation of the donor lung, recipient transplant surgery and ischemic time between lung retrieval from the donor and reperfusion in the recipient. Ischemia-reperfusion injury (IRI) is one of the most well-defined and important contributing factor to primary graft dysfunction (PGD). PGD is the most serious early complication occurring within the first 3 days after lung transplantation, with an incidence of 11-25%. (Christie, Carby, et al. 2005; Christie, Kotloff, et al. 2005; Porteous, Diamond, and Christie 2015) Severe PGD is associated with a 20-30% mortality rate in the first month after transplantation and a worse long-term survival. (Whitson et al. 2007; Kreisel et al. 2011) There is currently no effective clinical therapy used to prevent or treat PGD. (Porteous, Diamond, and Christie 2015) The specific pathophysiologic mechanisms that contribute to PGD are multiple and represent an active area of investigation.

During IRI, endothelial and other cells generate reactive oxygen species (ROS). Alveolar macrophages produce pro-inflammatory cytokines, including TNFα that promotes the production of other pro-inflammatory cytokines by epithelial cells. Neutrophils are activated and recruited in response to the high levels of circulating pro-inflammatory cytokines and chemokines. Neutrophil infiltration leads to the release of more cytokines, neutrophil elastase, and formation of neutrophil extracellular traps (NETs). (Laubach and Sharma 2016) The IRI itself also activates innate immune responses and promotes a pro-inflammatory microenvironment that predisposes the lung to the development of the clinical manifestations PGD which directly contributes to morbidity and mortality after transplant. (Ehlers 2014; Bergin et al. 2010; Jonigk et al. 2013)

*Alpha-1-antitrypsin (A1AT)* is a well-known protein that has been the subject of extensive research over the last 30 years. It is a serine protease inhibitor, mainly produced by the liver that acts as an
acute phase reactant during an infection or inflammatory response. Patients with A1AT deficiency can develop emphysema as a consequence of a protease-antiprotease imbalance in the lung. Synthetic recombinant A1AT has been effectively used intravenously as augmentation therapy in A1AT deficiency patients, showing a broad safety profile with limited and well tolerated side effects. (Petrache, Hajjar, and Campos 2009) It is a well-established clinical standard of care. Recent findings have suggested that A1AT can have a wider spectrum of biological and immune modulatory effects. (Wanner, Arce, and Pardee 2012)

While many agents have been studied experimentally to reduce IRI, few if any, have made it to clinical application. In fact, to date there is no agent approved to prevent or treat PGD and supportive care is the norm for care of patients with severe PGD in the intensive care unit. Biologically, A1AT is a promising agent to attenuate or prevent IRI. We therefore set out on a systematic exploration (detailed below) of this agent in models of escalating complexity, going from simulated IRI in a human pulmonary epithelial cell culture model (Gao et al. 2014), to a warm IRI hilar clamping rat model (Gao et al. 2014), to a rat single lung transplant survival model, and then to a pig acute transplant model (Iskender et al. 2016). In each of these models, we have shown benefit of A1AT in ameliorating and preventing IRI after lung transplantation using a drug that is already clinically approved for use in humans. The goal of the current study was to evaluate A1AT in the final preclinical study – a large animal (pig) survival lung transplant model to determine dosing regimen, safety and clinical efficacy. The importance of this study lies in the fact that with the results obtained herein, we believe we are ready to translate this agent to the bedside in a trial of A1AT to ameliorate reperfusion injury in human lung transplantation.

In brief, we have published several studies demonstrating the positive effects of A1AT in reducing IRI by inhibiting neutrophil elastase, as well as reducing pro-inflammatory cytokines, such as IL-
An epithelial cell culture model showed that A1AT was able to prevent deleterious cell morphology changes after 18 hours of cold ischemic time and 4 hours of reperfusion. A1AT also reduced induced cell death via caspase-3 inhibition and reduced pro-inflammatory cytokine release in vitro including IL-6 and IL-8; A1AT additionally inhibited IR-induced loss of cell viability. In a rat model, after 90 minutes of warm ischemia and 120 minutes of reperfusion, A1AT improved lung oxygenation compared to controls. A1AT treatment also reduced the lung injury score, reduced neutrophil infiltration in the lung tissue and IRI-induced lung epithelial apoptosis. A1AT treatment significantly reduced plasma levels of IL-1α and TNFα. In order to advance on the path towards clinical application, we further tested the effect of A1AT in a more relevant in vivo model – a large animal acute lung transplant model. We tested the effect of A1AT on IRI using a pig left single transplant model with 4 hours of reperfusion. Treatment with A1AT significantly improved allograft gas exchange, lung edema (wet-to-dry weight ratio) and lung permeability index was markedly lower in the treatment group. Inflammation (IL-6 and IFN-γ levels) was also significantly lower in the treated animals. In the complex field of lung transplantation, one of the major challenges has been the lack of translation of basic science discoveries to clinical settings. The current FDA drug development guidelines require demonstration of positive effects in more than one animal species. When trying new drugs that have demonstrated to be promising in small animals, large animals are the necessary before human trials. A pig 3-day survival model as the ultimate pre-clinical study, will help us understand if A1AT treatment would be beneficial in the clinical lung transplant setting.
In this study, we used a pig lung transplant model to test the safety and clinical effect of A1AT administered to recipients starting just prior to reperfusion, with specific emphasis on clinical outcomes.

4.3. Material and Methods

Male domestic Yorkshire pigs (25–35 kg) were utilized in an experimental protocol approved by the Animal Resource Centre at Toronto General Hospital Research Institute, University Health Network, Toronto, Canada. Animal care was in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

A1AT dosing

A1AT has been used for decades as augmentation therapy for patients with A1AT deficiency, and it is well-known to be safe; (Petrache, Hajjar, and Campos 2009) however, the therapeutic target levels in these patients are in the range of the normal/subnormal biological levels (normal human serum concentration 0.9-1.74 g/L). As A1AT is an acute phase protein, the serum concentrations increase 2-4 fold during systemic inflammation. In our preliminary studies using ex-vivo lung perfused pig lungs, we established that a perfusate concentration of 3 g/L was sufficient to elicit the protective effects of A1AT. In our dose-finding experiments, we found that with the dose of 250 mg/kg, A1AT levels achieved therapeutic levels but A1AT was consumed very quickly during the first 24 h post-reperfusion. The explanation to this consumption could be that during inflammation A1AT is known forms a 1:1 protease-antiprotease complex in order to inactivate the protease, acting like a “suicidal protein” (Wanner et al. 2015). We therefore realized that a significantly higher dose of A1AT will be required for optimal therapeutic effect. We found that administration of A1AT at a dose of
500 mg/kg (which is higher than the usual clinical replacement therapy dose of 60-240 mg/kg) was well tolerated and resulted in no adverse effects in the recipient animals, as measured by hematological, kidney and liver function profiles. Dosage was determined based on a pharmacokinetic study. Dosage was determined based on a pharmacokinetic study (See Chapter 4.6. Appendix) which also confirmed that repeating this dose of 500mg/kg q 24h over the course of 72 hours was an effective way to keep the systemic levels of A1AT in our target range.

**Experimental groups**

Recipient pigs were randomly assigned to the treatment or control groups. The operating team were completely blinded to the study drug, and all analyses were performed in a double-blinded fashion. Recipients in the treatment group (n=6) were treated intravenously with 500 mg/kg human A1AT (Zemaira®, CSL Behring, King of Prussia, PA). Control animals (n=6) received an equivalent volume of placebo (normal saline). The duration of the infusion was 60 minutes and it was completed approximately 10 minutes before reperfusion.

**Lung transplantation and survival model**

We have previously published the detailed methods for the pig lung transplant 3-day survival model that was used in this study. In brief, pigs, received pre-induction sedation with intramuscular ketamine (40 mg/kg; Vetoquinol, Lavaltrie, QC, Canada) and midazolam (0.3 mg/kg; Fresenius Kabi Canada Ltd., Richmond Hill, Canada). Induction was performed with inhaled isoflurane (3-5%; Pharmaceutical Partners of Canada, Richmond Hill, Canada). Topical lidocaine was sprayed in the glottis (Lignocaine (10 mg/0.1 mL) 3 pumps sprays of 10 mg non-aerosol topical anesthetic; Astra Zeneca; Macclesfield, UK), and animals were intubated with a 7.5 size endotracheal tube. Maintenance anesthesia consisted of intravenous propofol (10
mg/mL; PharmaScience Inc., Montreal, Canada) and remifentanil hydrochloride (1 mg/100mL PMS-Propofol; Teva Limited, Toronto, Canada).

Donor animals were submitted to organ procurement following standard technique, described elsewhere. The donor lungs were subjected to 24 hours of cold ischemic preservation before transplantation. Left single-lung transplantation was performed according to the previously described technique. One hour after reperfusion, a blood sample was taken from the upper and lower pulmonary veins of the grafted lung to evaluate graft function. An analgesic intercostal block with bupivacaine (0.5% (5 mg/ml); Sensorcaine, Astra Zeneca, Mississauga, Canada) and lidocaine (100 mg/5 mL) (7 ml; Lidocaine HCl Injection USP, Alveda Pharmaceuticals Inc, Toronto, Canada) was performed. The pleural cavity was drained with a 24 Fr chest tube connected to an ambulatory drainage valve (Pneumostat, Atrium Medical Corp., Hudson). Postoperative analgesia was carried out with fentanyl patches (50 μg/h) and intramuscular long acting buprenorphine (0.01 mg/kg; Vetergesic®: Champion Alstoe Animal Health, Whitby, Canada). Antimicrobial prophylaxis was administered using ceftazidime (1 g/q12h; Pharmaceutical Partners of Canada, Richmond Hill, Canada) and ciprofloxacin (Ciprofloxacin (2 mg/mL in 5% (50 g/L) Dextrose Solution; Sandoz, Boucherville, Canada; q12h). The immunosuppressive regimen consisted of cyclosporine (5 mg/kg; Novartis Pharma Canada Inc., Dorval, Canada) and Methylprednisolone (1mg/kg/day, Solu-medrol®; Pfizer; Kirkland, Canada). As antithrombotic prophylaxis, enoxaparin sodium (30 mg/q24h; Lovenox®, Sanofi-Aventis, Laval, Canada ) was given.

**Post-transplant management**

After reperfusion, the lungs were ventilated for 10 minutes according to our protocol, with an FiO₂ of 60% using pressure control ventilation of 15 cmH₂O, and a positive end-expiratory pressure
(PEEP) of 5 cmH₂O. The respiratory rate was adjusted according to the pCO₂ to maintain values between 35 and 45 mmHg. One hour after reperfusion, a blood sample was taken from both pulmonary veins, and a bronchoscopy was performed to assess if there were any signs of edema from the lungs. If the bronchoscopy was clear, and the pO₂ of the graft was >200 mmHg, the chest wall was closed. In case of edema and/or pO₂ <200 mmHg, the pig was left sedated for another hour, and bronchoscopy and pulmonary vein pO₂ were reassessed. According to our protocol, 1 pig in each group was sacrifice at this point because of pO₂ <200 mmHg and signs of severe lung edema. After closing the neck incision, the arterial and venous neck lines that had been placed before the transplant were secured in order to maintain access to arterial blood for blood gas assessment and the venous line for post-operative intravenous medication. Propofol and fentanyl infusions were slowly reduced starting 1 hour after reperfusion. Buprenorphine (3 ml) was given intramuscularly after the pig was positioned in the prone position. On day 3 after transplantation, the animals were anesthetized as previously described, and a median sternotomy was performed. The right pulmonary artery, right atrium and right bronchus were clamped for 5 minutes followed by arterial blood gas sampling for isolated lung graft functional assessment.

**Immediate post-transplant clinical assessment**

After the pig started to breathe spontaneously the ventilator was changed to pressure-support mode and the ventilator support was weaned slowly. The endotracheal tube was left in place as long as the pig tolerated it. The pig was extubated once breathing spontaneously without any support from the ventilator, and when the pig was coughing or fighting the tube. The time of extubation was recorded. Oxygen (3-5 L/min O₂) was administered via an oxygen mask. The pig was then returned to the holding pen. The saturation was continuously monitored with a portable oximeter attached to the pigs ear. Supplementary oxygen was provided during transportation and in the holding area.
via facemask. O$_2$ flow was decreased incrementally every 5-10 minutes if O$_2$ saturation $>$ 85% until weaned off.

Until the recipient animal was able to maintain saturation of O$_2$ $>$ 85% on room air. This time point was recorded as “time the pig was weaned of oxygen”. The time (in minutes) that the pig had to remain on supplementary oxygen was documented as a primary clinical outcome. Recipient pigs were closely monitored for hemodynamic and respiratory function, and a biochemical safety analysis was performed, including hematologic, hemostatic and serum biochemistry testing. The clinical status of the pig was also recorded every 6-8 hours. The animal weight was measured and recorded pre-transplant and 3 days after transplantation.

**Sample collection**

Systemic blood samples were collected 10 minutes after reperfusion, every hour for the first 6 hours after reperfusion, and then daily from the internal jugular vein indwelling catheter (9.5 Fr TPN Catheter set, Cook Medical, Bloomington, IN) for analysis of hemoglobin, white blood cell count and differential, platelets, urea, creatinine, electrolytes, alanine transaminase (ALT), and aspartate transaminase (AST). Lung biopsies were collected at day 3 post-transplantation; one portion of the biopsy tissue was snap frozen in liquid nitrogen and stored at -80 $^\circ$C, and the other was injection-inflated with formalin and prepared for histopathological assessment.

**Neutrophil Elastase and inflammatory response**

Neutrophil elastase analysis was performed using a porcine neutrophil elastase enzyme-linked immunosorbent assay kit (MyBioSource California, USA). For tissue cytokine analysis, frozen lung tissue was homogenized as previously described (Yeung, Wagnet, et al. 2012). The systemic inflammatory cytokines (IL1$\beta$, IL6, IL 8, and TNF$\alpha$) and nuclear factor kappa-light-chain-
enhancer of activated B cells (NFκβ) were measured in plasma and tissue homogenized samples using polymerase chain reaction (PCR).

**Apoptotic cell death**

Tissue sections were stained with deoxynucleotide transferase-mediated deoxy uridine triphosphate nick-end labeling (TUNEL) to assess apoptosis (In Situ Cell Death Detection Kit, TMR red; Roche Waltham, USA). An inverted fluorescence microscope was used to photograph 10 to 12 random fields per slide. TUNEL positive cells were counted by a blinded observer using ImageJ software (National Institutes of Health) and expressed as the percentage of total cells.

**Statistical analysis**

All results are expressed as mean ± standard error of the mean. Mann–Whitney nonparametric testing was used for comparisons between two groups. Statistical analyses were performed with Prism 7 software (GraphPad Software, Inc., La Jolla, CA). Differences were considered statistically significant when the p-value was < 0.05.

4.4. Results

**A1AT improves clinical outcome after transplantation**

A1AT administration resulted in quicker clinical recovery. The pigs receiving A1AT treatment demonstrated a significantly decreased time to weaning off supplemental oxygen (20.6+/−9.3 min) when compared with controls (125+/−25 min) (P= 0.022) (Figure 12A).

All pigs were clinically well 24 hours after transplant and remained well until sacrifice at 72 hours. Treated pigs were standing 3 hours after extubation in comparison with untreated pigs that were lying down at the same time point. They showed normal activity and appetite throughout the 3-day period. (Figure 12B). The physiological parameters from the transplanted lung were measured
72 hours after transplantation by clamping the native lung hilum. Both groups showed very good oxygenation as well as good static and dynamic compliance, overall the function seemed better in the A1AT group, but the differences were not statistically significant (Figure 12C).

(A) Time in minutes until the pigs were able to be weaned of oxygen

(B) Photograph of recipient pigs taken 2 hours after extubation
Figure 12 Clinical Recovery and Lung Graft Functional Parameters

(A) Time in minutes until the pigs were able to be weaned of oxygen (O2 sat > 85% on room air) after extubation. The pigs who received A1AT treatment demonstrated a significantly decreased time to weaning of supplemental oxygen (21 ± 9 min vs. 125±25 min in control, p= 0.022. (B) Photograph of recipient pigs taken 2 hours after extubation (taken in a blinded fashion). The treated group were independently standing and walking at that time while pigs in the control group were lying down, most of them still on supplementary oxygen. (C) The physiological parameters from the transplanted lung measured 72h after showed good oxygenation as well as good static and dynamic compliance in both groups, overall the function seemed better in the A1AT group, but the differences were not significant.

Treatment group exhibited lower neutrophil elastase activity in lung tissue collected 72 hours after transplant compared with control group (Figure 13A).

Pro-inflammatory cytokines were measured in lung tissue samples using polymerase chain reaction (PCR) three days after transplantation. We did not find any significant differences between the treatment and the control groups (Figure 13C).
NFκβ measured with PCR and TUNEL-positive cells counted and expressed as the percentage of total cells from stained tissue sections were reduced in the A1AT treated group, without achieving statistical significance (Figure 13B).

(A) Neutrophil Elastase

(B) Cell Death (Tunel Staining)
(C) Pro-Inflammatory Cytokines and NFκβ in Lung Tissue at 72h (PCR)

Figure 13 Neutrophil Elastase and Cell Death and Inflammation—Lung Injury: Pro-inflammatory Cytokines

(A) A1AT treated pigs demonstrated significantly lower neutrophil elastase in lung tissue samples (p= 0.041). NFκβ (iPCR) (B) Tunel Staining. (C) Pro inflammatory cytokines measured in lung tissue 3 days after transplant didn’t show any significant differences.

A1AT treatment showed no evidence of systemic toxicity

There were no signs of hematological, renal, or hepatic toxicity observed in the A1AT treated group compared to controls (Figure 15). All the parameters that were tested in blood 1 hour after reperfusion and every 24 h for three days were into normal range and there was no difference between control and treated pigs.
Figure 14 Recipient Systemic Safety and Toxicity Profile of A1AT

(A) Hematologic evaluation of blood samples revealed similar stable hemoglobin levels and white blood cells. Platelet counts were significantly higher in the A1AT group, but still in the normal range. (B) Assessment of kidney function reveals similar (normal) levels of creatinine and urea. (C) Liver function, expressed by Bilirubin, AST and ALT levels, were also normal and similar in both groups.
4.5. Discussion

In this study, we examined A1AT treatment in a pig single lung transplant survival model developed by our group to test novel therapeutics to ameliorate or prevent IRI and PGD in the first three days after transplantation. (Machuca, Cypel, Bonato, Yeung, Chan, Juvet, Zehong, et al. 2017; Pierre et al. 1998; Iskender et al. 2016) This model focuses on ischemia reperfusion injury. After three days, infection and immunologic rejection play a bigger role in the lung injury profile and confound the picture.

In the present study, we show that A1AT facilitates post lung transplant clinical recovery in a large animal lung transplant model. After obtaining extensive biologic and mechanistic evidence for the benefit of A1AT in IRI in previously published studies, we used a pig lung transplant 3-day survival model as the penultimate pre-clinical study prior to embarking on a human clinical trial to prevent PGD with A1AT. These clinically beneficial results are consistent with the prior experimental studies and are encouraging; they suggest that administering A1AT to the recipient before reperfusion may lead to less lung injury and a faster recovery of patients after transplant.

As a limitation in this study, the cytokines and apoptosis were measured 3 days after transplantation when all the pigs (controls and treatment) had recovered from the surgery. We used a 24 h cold ischemia time as the baseline injury for a clinically relevant time frame. We recognize (and observed) that when trained surgeons perform the operations, this type of injury may not provide a sufficiently damaged baseline to show significant differences between controls and treatments, and that the pigs in the study did not develop PGD.

These results are important because, thanks to key advancements in lung preservation, surgical technique and post-transplant management more transplants are performed worldwide each year. (Christie et al. 2012) However, one of the main limitations to lung transplantation remains the shortage of donor lungs, which increases the waitlist mortality. The utilization rate of lungs from
multi-organ donors is still low. (Halldorson and Roberts 2013; Klein et al. 2010) One of the main concerns about using suboptimal lungs is the development of PGD, a condition that is known to be responsible for a high (20-30%) mortality rate in the first month after lung transplantation, and that is also known to be associated with the development of chronic lung allograft dysfunction and lower long term survival rates. (Lee, Christie, and Keshavjee 2010; Porteous, Diamond, and Christie 2015) Thus, finding a means to prevent or alleviate the development of PGD, by reducing the contribution of IRI, could help patients to recover and decrease other complications and mortality.

The finding that A1AT helped recipient pigs to recover quickly after surgery in this survival model supports the idea of using A1AT in the setting of acute lung injury. Accelerated lung recovery after transplant surgery would likely lead to a decreased recipient length of time on mechanical ventilation and likely a shorter ICU and hospital length of stay, overall making the recipient less likely to develop further complications.

Overall, we show that A1AT is safe, well tolerated, and clinically useful in the setting of lung transplantation. Faster recovery and weaning off supplemental oxygen dependence indicates that A1AT could potentially play a role at reducing length of mechanical ventilation and ICU length of stay. There are still further questions to be studied to elucidate the underlying mechanisms leading to the faster clinical recovery after lung transplantation. Testing a new drug in different models and even different species is essential to understand how it works specially before moving to a human clinical trial. The three day pig lung transplant survival model is an good pre-clinical model and is the closest we can get to the actual in vivo setting, before being able to test the drug in humans.

Biologically, A1AT is a promising agent to attenuate or prevent severe PGD after lung transplantation. We have systematically studied this drug in multiple models in various species
and the results have demonstrated a positive effect. The goal of the current study was to evaluate A1AT in the final preclinical study – a large animal (pig) survival lung transplant model to determine the dosing regimen, safety and clinically relevant efficacy. With these results, we believe we are ready to translate this agent to the bedside in a trial of A1AT to ameliorate reperfusion injury in human lung transplantation.

4.6. Appendix

4.6.1. Pharmacokinetic Studies

The current clinical practice in A1AT deficient patients aims to reach 0.5 g/L of total serum A1AT concentration. The existing recommendation is to use between 120 and 240 mg/Kg biweekly or monthly. (Soy et al. 2006) Since our study was related to A1AT as a strategy to decrease PGD, not just by inhibiting neutrophil elastase but also by lowering inflammation and cell death related to reperfusion injury, we aimed for supra-therapeutic levels. In order to find the ideal dose of A1AT pharmacokinetic studies were performed

**Material and Methods**

Central jugular vein placement. Under anesthesia pigs were intubated. The pig were positioned with the left forelimb down and away from the head to ensure the neck was completely extended. A 6 cm neck incision was made, 2 cm left from the mid line. Dissection was performed until the jugular vein was reached and encircled with two 2-0 silk ties. A clamp was placed on the proximal vein and the distal end was ligated. The internal jugular vein indwelling catheter (9.5 Fr TPN Catheter set, Cook Medical, Bloomington, IN) was placed proximally. The catheter was secured and the wound was closed. Pigs were divided in 3 groups (n=2 in each group):
1. Single left lung transplant group (n=2): single left lung transplants were performed after 24 hours of cold ischemia. The pigs received 240 mg/kg of human A1AT before reperfusion intravenously.

2. No transplant group + 240 mg/Kg (n=2): Pigs received 240 mg/Kg of human A1AT

3. No transplant group receiving 500 mg/kg (n=2): Pigs received 500 mg/Kg of human A1AT

**Results**

In the transplant group the median hA1AT levels 1 h after A1AT administration were 0.23 g/L (± 0.04). The levels dropped very quickly post-transplant and were almost undetectable after 24 hours. (Figure 15) In the 240 and 500 mg/Kg groups the initial levels were around 1.2 g/L and 0.4 at the end of the 3 days (Figure 16).

![Alpha 1 Antitrypsin Levels](image)

*Figure 15 A1AT plasma levels from pigs that receive 240 mg/Kg before reperfusion*

A1AT levels measured Every 24 hours until 72 h after transplantation.
A1AT levels measured Every 24 hours until 72 h after transplantation.

We conjectured that circulating levels of A1AT is determined by the dose administered and that the hA1AT could be consumed by the transplanted damaged lungs. Biochemical safety analysis, including hematologic, biochemical, and hemostasis screening tests were performed and no detrimental effects were observed.

We then chose the higher dosage of 500mg/kg and decided to repeat the dose every 24 hours for the three days post-transplant period in a blinded fashion. With the new dosage, we achieved supra-therapeutic and stable levels of A1AT in all treated cases. (Figure 17)

Figure 16 A1AT Levels in Treated Non-Transplanted Pigs (Pharmacokinetic Studies)

Figure 17 A1AT Levels During Time in Treated Transplanted Pigs remained supra-therapeutic and stable
Chapter 5. Alpha 1 Antitrypsin Treatment During Ex Vivo Lung Perfusion Improves Human Lung Function by Protecting Lung Endothelium

This Chapter has been modified from an article that is currently under review for publication.
5. Alpha 1 Antitrypsin Treatment During Ex Vivo Lung Perfusion Improves Lung Function by Protecting Lung Endothelium

5.1. Abstract

**Introduction:** Only 20-40% of the potential donor lungs end up being used. The underutilization of potential donor lungs is explained by the concern that the recipient could develop one of the most important complications early after transplantation: primary graft dysfunction (PGD). PGD has a 20-30% mortality rate and occurs in 11-25% of the transplanted patients. There is currently no approved therapy to prevent or treat PGD. Finding a treatment that prevents PGD from occurring would increase the donor pool significantly and reduce wait list mortality. Ex vivo lung perfusion has shown to be an ideal platform to evaluate potential therapeutics before transplant.

Alpha 1 antitrypsin (A1AT) is a serine protease inhibitor and its most specific substrate is neutrophil elastase (NE). Recent studies have shown that A1AT may have anti-inflammatory properties. The aim of this study was to determine the effect of A1AT given during EVLP in the lung endothelium.

**Material and Methods:** Lungs from 8 human multi-organ donors that were rejected for clinical transplantation were used. The lung block was separated, and each lung was placed on a separate EVLP circuit, and randomly assigned to either receive A1AT or placebo. EVLP was run for 12 h. Lung function was assessed every hour. Cytokines and soluble molecules in perfusate were measured at 1 h and every 3 h until the end of EVLP. Perfusate lost was recorded every hour as an indirect sign of vascular permeability. Wet-dry ratio from lung tissue that were taken before putting the lungs in the circuit and at the end of EVLP, to assess lung edema. Zonula occludens protein-1 (ZO-1) was evaluated with immunofluorescent staining to assess tight junction integrity.
Results: A1AT group showed significantly better lung function. Perfusate loss during EVLP was significantly lower in the treatment group (p= 0.01). Wet-dry ratio after EVLP was lower in the A1AT treated lungs. A1AT had an effect decreasing ET-1 in the EVLP perfusate. Treated group showed increased ZO-1 expression (p= 0.05).

Conclusions

The use of A1AT during EVLP was able to improve human donor lungs that were rejected for transplantation by protecting the endothelial cell function.

5.2. Introduction

Since the first successful lung transplant performed more than 30 years ago, the number of lung transplants performed each year has been exponentially increasing. The number of offered lungs, however is not even close to the number of patients waiting for lungs to become available. This imbalance between patients on waitlist and donor lungs has been translating into more patients dying on waitlist. One of the reasons of this shortage of donor lungs is the underutilization of the organs. From the potential donor lungs, even the most aggressive centers just use 20-40%. Lungs are known to suffer various injuries that can compromise the lung function during the process of brain death or during the hospital stay. The use of marginal donor lungs has been correlated to worse outcomes and is associated to the development of PGD. PGD is a severe form of acute lung injury that occurs during the first 72 h after transplantation and represents the most serious complication during the initial post-transplant phase. The exact mechanism by which PGD is developed remains unknown, but IR injury plays an important role. IR injury is a fast and multifaceted inflammatory response that entails endothelial dysfunction.
Endothelial function is crucially involved in physiologic immunity at different stages including recruitment of leukocytes, angiogenesis and tissue repair.

Ex vivo lung perfusion has shown to be an excellent platform to evaluate lungs before transplantation and to potentially treat injured lungs. (Cypel and Keshavjee 2016; Cypel et al. 2008)

A1AT is a serine protease inhibitor produced by the liver, alveolar cells and other immune cells. Its most specific substrate is neutrophil elastase. (Crystal et al. 1989; Travis and Salvesen 1983) A1AT concentration increases as a response to inflammation. Recent studies have shown that A1AT may have anti-inflammatory and anti-apoptotic effects in different settings. A1AT treatment before in a lung transplant setting has shown to be safe and well tolerated in a previous study performed by our group. (In press) Our hypothesis is that A1AT may have an effect on lung function by inhibiting inflammation and by protecting the lung endothelium. Testing A1AT in human lungs before clinical application is an important step of translation.

5.3. Material and Methods

5.3.1. Material and Methods Human Lung Study

Experimental Design

Lungs from 8 human multi-organ donors (brain death or DCD) that were offered to the Toronto Lung Transplant Program and were determined to be clinically unsuitable for transplantation for any reason were used. All donors had a research consent signed. The lungs were preserved according to Toronto Lung Transplant protocols using cold low-potassium dextran solution (Perfadex®, XVIVO Perfusion, CO, USA) as flush solution and then maintained at 4 °C for transport. The lung block was separated, and right and left lungs were placed on two separate
EVLP circuits. Each lung was randomly assigned to either receive A1AT (Zemaira®, CLS Behring) treatment or placebo through the EVLP perfusate. For the treatment group 10 mg/ml of A1AT was added to 1.5 L of Steen® solution (XVIVO Perfusion, Gothenbutg, Sweden). Lungs were maintained for 12 h at body temperature in the XVIVO system.

**EVLP**

Immediately after the donor lungs arrived at the research laboratory, the lung block was separated and both lungs were cannulated, following the Toronto EVLP method described previously. In summary the left atrial (LA) cuff was attached to the LA cannula with a 4-0 polyprolene running suture. The pulmonary artery (PA) canula was inserted to the PA making sure it was placed proximal to the bifurcation and was secured with silk ties. The trachea was clamped above the carina, the stapler line that was placer during the donor retrieval in the trachea was opened, an endotracheal tube was inserted and secured with 2 silk ties. Endotracheal tube was clamped and the tracheal clamp released. A retrograde flush was performed using 1 L of low potassium dextran solution, making sure there was no leakage around the cannulas. The circuit was primed with 1.5 L of Steen® solution (with or without A1AT), 500 mg methylprednisolone (Solumedrol; Sandoz Canada, Boucherville, Canada), 3,000 IU of unfractionated heparin (Organon, Canada) and 1 g of cefazolin (Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada) were added to each circuit.

Following the Toronto EVLP protocol, pulmonary recruitment maneuvers were performed every hour (30 minutes before assessment), by increasing the tidal volume by 100cc with following by inspiratory hold maneuvers up to 25 cm H2O (and a maximum of 15 cc/kg) for ten seconds. The physiological assessments were performed every hour. Before each assessment the ventilatory parameters were changed from maintenance (7 mL/kg of tidal volume, PEEP 5 cm H2O and 7 of
respiratory rate/min), to assessment mode (10 ml/kg tidal volume, 10 breaths per minute and FiO₂ 100%). Five minutes later, all the parameters are recorded including: static compliance, dynamic, compliance, PA pressure, LA pressure, peak airway pressure, and plateau pressure. Compliance (dynamic and static) and peak airway pressure were recorded from the ventilator ( ). Pressures (pulmonary venous pressure and pulmonary artery pressure) were measured using a standard pressure monitor. Delta pO₂ was calculated using the pre- and post-membrane perfusate pO₂. After assessment all parameters were changed back to maintenance mode. After the first hour of EVLP, 250 mL of circulated perfusate was removed and replaced with 250 mL of fresh solution (with or without A1AT). Afterward, 100 mL was exchanged every hour until the end of EVLP. The lungs were perfused for 12 h or until they failed and EVLP had to be stopped. Failure was determined by massive edema fluid evidenced inside the endotracheal tube and the need to add more than 250 cc of perfusate solution to the circuit in less than 30 min in order to maintain a minimum of 200 cc of perfusate in the reservoir, and peak airway pressure above 25 cmH₂O

**Tissue Biopsies and Perfusate Samples**

EVLP perfusate was collected every hour before the perfusate exchange. Lung tissue samples were taken before EVLP and then every 3 hours. Samples were taken from the lower lobes during the EVLP and from all lobes at the end of EVLP; trying to be as consistent as possible. Perfusate samples were divided in 2, half of the sample was aliquoted and immediately snap-frozen and stored at -80°C until analysis, the other half went to cell suspension process. Lung tissue biopsies were divided into 3 specimens. First specimen was immediately snap-frozen and stored at -80°C until analysis. The second specimen was processed for histologic assessment by fixing it in 10% buffered formalin initially and then embedding it in paraffin and sectioned onto slides; slides were stained with hematoxylin and eosin (HE) and examined in a blind fashion for lung injury by a lung
pathologist blinded to the study. The third 1.5 x 1.5 cm tissue specimen that was taken from the dependent parts of the lower lobes was used to calculate the wet to dry ratio in order to evaluate lung edema as previously described.

Homogenization of Lung Tissue

Frozen lung tissue was homogenized and crushed into powder using a cold mortar and a pestle. Powered lung tissue (50 mg) was put into a microcentrifuge tube containing 1 mL of lysis buffer, tissue and buffered were centrifuged at 10,000 rcf at 4 degree Celsius for 15 min. The supernatant was aliquoted and stored at -80 degrees Celsius until needed for analysis.

Tissue dissociation protocol

Lung tissue were minced and enzymatically digested with collagenase A (Roche) and DNase I (Sigma-Aldrich) for 10mins, before being mechanically dissociated with a gentleMACS™ dissociator (Miltenyi). Dissociate were filtered with a 70um cell strainer, and spun down at 400g. Red blood cells were lysed using Red Blood Cell Lysis Buffer (NH₄Cl, KHCO₃, EDTA). Cells were washed with PBS with 2% HI-FBS and 2mM EDTA, counted, and frozen with FBS/10% dimentyl sulfoxide

A1AT Levels in Perfusate

Concentration of A1AT in perfusate were measured by an enzyme-linked immunoassay kit (Human Alpha 1 Antitrypsin ELISA Kit (SERPINA1, ab189579); Abcam, Toronto, ON, Canada); following the manufactures directions .

Cytokine Analysis

- Multiplex Assay

Perfusate levels of cytokines, chemokines and growth factors at 1, 6 and 12 h were measured by a Bio-Plex Pro Human Cytokine 27-plex Assay kit (Bio-Rad Laboratories, Mississauga, Canada), following the manufacturer’s protocol. The analytes were read with
a Luminex 100 analyzer (Luminex, Austin, CA), and the data were analyzed using Bio-Plex Manager 6.0 (Bio-Rad Laboratories).

- **Single ELISA**

  Concentration of IL-8, IL-6 and adhesion molecules (ICAM, VCAM and E Selectin) in perfusate were measured using single enzyme-linked immunoassay kits according to manufacturers instructions. IL-8 was measured Quantikine ELISA kit Human IL-8/CXCL8 (R&D Minneapolis, MN). IL-6 Quantikine ELISA Human IL-6 (R&D Minneapolis, MN). The sICAM-1 was measured by the Human ICAM-1/CD54 Allele-specific Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Minneapolis, MN), sVCAM-1 was measured by a human VCAM-1 ELISA kit (Boster Biological Technology Co., Ltd., Pleasanton, CA), and E selectin was measured with a human E-Selectin ELISA kit (RayBiotech, Inc., Norcross, GA).

**NE-A1AT Complex**

At time of analyses, perfusate samples were thawed on ice once. Perfusate NE-A1AT complexes were quantified using a capture enzyme-linked immunosorbent assay (ELISA) commercially available (Human PMN elastase ELISA kit, Abcam, Cambridge, MA). Briefly, samples were added to plates precoated with PMN Elastase specific antibodies along with HRP-conjugated PMN elastase detection antibody. After incubation, unbound proteins were washed out and TMB was used to visualize the enzymatic reaction. The capture antibody used was specific to PMN elastase, and the detection antibody was specific to A1AT, making the ELISA detection specific to the NE/A1AT complex.

**Permeability Studies**

- **Perfusate Loss**
Perfusate levels in the reservoir was assessed every hour, before addition and sampling. Perfusate lost was measured hourly for the 12 hours.

- **Wet-Dry Ratio**

  Lung tissue sections were weighed before EVLP and at the end of EVLP, dried in an oven at 80°C for 72 h and re-weighed. Wet weight was divided by dry weight to calculate the W/D ratios.

- **Tight junction integrity**

  Zona occludens protein-1 (ZO-1) tight junction integrity was analyzed using Immunofluorescent staining. 4 μm of Paraffin-embedded sections were deparaffinized and were followed by antigen retrieval in boiling 0.01 M citrate buffer (pH 6) for 20 minutes. Then the sections were blocked with 3% Hydrogen Peroxide solution for 1h, protein blocking buffer for 1h. Incubated overnight at 4°C with ZO-1 antibody ( Invitrogen, Catalog number: 61-7300) at 1:400 dilution. After washing with PBS-0.1% Tween the sections were incubated with poly-HRP-conjugated goat anti rabbit secondary antibody ( Thermo Fisher : Catalog number: B40925 at room temperature for 1h. After washing , apply Alexa Fluor 594 tyromide working solution to the section abd incubate at room temperature for 6min. Then add stop solution to stop HPR reaction, wash and mounted with SlowFade Gold antifade reagent with DAPI (Invitrogen, Catalog Number S36938). Images were acquired with Yokogawa Spinning-disk Confocal microscope.

  The staining intensity in the cell membrane of alveolar cells was evaluated with a semiquantitative scoring system: no staining (−), low staining (+), intermediate staining (++), and strong staining (+++). The results were evaluated by two independent investigators and averaged.

**Statistical Analysis**
Measurements for lung physiology were taken hourly for 12 hours or until lung failure, leaving 184 data points (8 participants X 2 lungs X 12 hours minus 8). The cytokine levels were measured 5 times over the course of 12 hours, giving a total of 78 data points (8 participants X 2 lungs X 5 timepoints). To address the within-subject, repeated measures design of the study, mixed-effects linear models were used to examine differences over time for lung physiology measures and cytokine levels in Perfusate. Included as fixed effects were sex, age, TLC, lung (L/R), and smoking status, in addition to the main effect of interest: A1AT treatment. An autoregressive covariance structure was assumed, but compound symmetry was used when that model did not converge. This method was sufficient to describe the differences in lung physiology measures. However, model fit statistics for cytokines indicated that residuals were not normally distributed and therefore that the p-values from these models would be incorrect. Instead, p-values were obtained from a permutation test on the A1AT categorical treatment effect. For each of the $2^8 = 256$ possible ways in which A1AT or control could be assigned within each pair of lungs, the mixed effects model was refitted and the t-statistic for the effect of A1AT treatment was calculated. This distribution of 256 t-values is what results when treatment is assigned at random to one lung in each pair. The t-statistic for the effect of A1AT treatment from the actual assignment was then compared to this distribution of t-statistics from all possible assignments, to check how unusual the observed data are when treatment is assigned at random. The p-value was computed as the proportion of possible assignments that had t-statistics larger than the actual one. Mean differences were presented as the measure of difference between treated and untreated lungs. All statistical analyses were performed with R 3.5.1 (R Core Team (2018). R: A language and environment for statistical computing. (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/).
5.3.2. Material and Methods Cell Culture Study

Cell culture

Human pulmonary endothelial cells (HPMEC) were obtained from Dr. Kirkpatrick. (Krump-Konvalinkova et al. 2001) Cells were cultured in HPMEC medium containing M199, 20% FCS, Glutamax (2 mM), Pen/Strep (100U/100ug/ml), heparin (50 ug/ml), and ECGS (50 ug/ml) at 37°C in a humidified incubator containing 5% CO2.

IR cell culture model

HPMEC cells were cultured at 37°C and 5% CO2 until confluent. Ischemia was simulated by replacing HPMEC medium with ice-cold cold low-potassium dextran solution lung preservation solution (Vitrolife, Englewood, CO), and cells were incubated for 6 h in a chamber filled with 50% O2 stored at 4 °C. Simulated EVLP reperfusion was performed by replacing the low potassium dextran solution with Steen®, and the cells were incubated at 37°C with 5% CO2 for 2 h, 4 h, and 18 h. Samples were taken at different time points, centrifuged at 10,000g for 5 minutes at 4°C to remove cellular debris, and the supernatant was frozen for subsequent analysis.

A1AT treatment

The drug was reconstituted according to the manufacturer’s manual. The three following concentration were used: 1, 5, and 10 mg/ml. After HPMEC cells reached confluency, cells were pre-treated with A1AT for 1h, and the media containing A1AT was removed, and cells were washed with PBS (Ca and Mg free). A1AT was added to Perfadex for 6 hour CIT and to Steen® solution during 2, 4, and 18 hours of reperfusion.
**Endothelin analysis**

Endothelin-1 (ET-1) was measured using human Endothelin Quantikine ELISA kit (R &D system, Catalogue number DET100) following the manufacturer’s protocol. Results were expressed in the concentration units of pg/ml.

**Statistical analysis**

Data analysis was carried out using GraphPad Prism 8.2.0. Significance was determined using an ordinary one-way ANOVA and Tukey’s multiple comparisons test. P-value less than 0.05 was considered significant. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** p < 0.0001).

**5.4. Results of the Experiments done in Human Lungs**

The donor characteristics from the 8 cases are summarized in Table 14.

---

**Table 14 Donor Characteristics**

<table>
<thead>
<tr>
<th>Case</th>
<th>Donor Cause of Death</th>
<th>Donor Type</th>
<th>TLC</th>
<th>Reason for being Declined</th>
<th>Other Relevant Donor Notes</th>
<th>A1AT-Treated Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Intracranial hemorrhage</td>
<td>DCD</td>
<td>7.76</td>
<td>Broncho aspiration</td>
<td>Treated lung purulent secretions and edema. Consolidation</td>
<td>Left</td>
</tr>
<tr>
<td>#2</td>
<td>Intracranial hemorrhage</td>
<td>NDD</td>
<td>7.04</td>
<td>Low pO2</td>
<td>Purulent secretion and consolidation treated lung. Smoker</td>
<td>Right</td>
</tr>
<tr>
<td>#3</td>
<td>Head and chest trauma</td>
<td>NDD</td>
<td>6.54</td>
<td>Heavy smoker + low pO2</td>
<td>Bilateral contusion and pneumothorax</td>
<td>Left</td>
</tr>
<tr>
<td>#4</td>
<td>Anaphylactic reaction</td>
<td>NDD</td>
<td>5.92</td>
<td>High airway pressure + low compliance</td>
<td>Right lung mild basal consolidation</td>
<td>Right</td>
</tr>
<tr>
<td>#5</td>
<td>Cardiac arrest</td>
<td>DCD</td>
<td>7.13</td>
<td>Emphysema</td>
<td>Heavy grow on S pneumoniae in BW Heavy smoker</td>
<td>Left</td>
</tr>
<tr>
<td>#6</td>
<td>Intracranial hemorrhage</td>
<td>NDD</td>
<td>8.75</td>
<td>Low pO2, Pseudomonas infection</td>
<td>Opacity on right lower lobe</td>
<td>Right</td>
</tr>
<tr>
<td>#7</td>
<td>Intracranial hemorrhage</td>
<td>NDD</td>
<td>7.58</td>
<td>Emphysema + low pO2</td>
<td></td>
<td>Left</td>
</tr>
<tr>
<td>#8</td>
<td>Intracranial hemorrhage</td>
<td>NDD</td>
<td>7.13</td>
<td>Emphysema + low pO2</td>
<td></td>
<td>Right</td>
</tr>
</tbody>
</table>
Summary of the characteristics of the 8 human donors rejected for clinical transplantation. Cause of death was intracranial hemorrhage for 5 cases, 1 traumatic injury, one case of cardiac arrest and 1 case of anaphylactic reaction. Two of the donors were donors after cardiac death and the other seven brain death donors. The reason for rejecting the lungs was different among the donors most of them had low pO₂.

5.4.1. A1AT Levels in Perfusate

Following the delivery of 10 mg/ml of A1AT in the perfusate, we expected to achieve A1AT levels of around 5 g/L. We were able to achieve supra-therapeutic levels of A1AT 1 h after EVLP start and during the 12 h of EVLP in the treatment group. A1AT levels in the treatment group (expressed in mean and standard deviation) were: 3.0750 (5.2967) after 1 h, 3.4277 (5.088) after 3 h; 5.9761 (6.5695) after 6 h; 6.64343 (6.2250) after 9 h of EVLP; and 11.028 (7.6948) at the end of the 12 h perfusion. A1AT levels remained undetectable in the control group at all time points. (Figure 18)

![A1AT Levels in Perfusate](image)

Figure 18 A1AT Levels in Perfusate

Supratherapeutic levels of A1AT were achieved during the 12 h EVLP by delivering 10 mg/mL of A1AT into perfusate. The A1AT levels in the control group were not detectable.
5.4.2. A1AT-NE complex in perfusate

A1AT treated lungs showed significantly higher NE-A1AT complexes in perfusate in comparison with the non-treatment group (p= 0.043). (Figure 19)

![NE-A1AT Complex in Perfusate](image)

Figure 19 NE-A1AT Complex
Treatment group showed significantly higher NE-A1AT complex measured by ELISA (p= 0.0043)

5.4.3. Effect of A1AT on lung physiology during EVLP

The A1AT group showed significantly better lung function exhibiting a higher ΔPo2/Fio2 and higher dynamic compliance and static compliance. See table 15 (Figure 20)

<table>
<thead>
<tr>
<th></th>
<th>LOWER</th>
<th>ESTIMATED</th>
<th>UPPER</th>
<th>p VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔPo2/Fio2</td>
<td>18.70</td>
<td>68.41</td>
<td>118.13</td>
<td>0.01</td>
</tr>
<tr>
<td>Peak Pressure</td>
<td>-4.60</td>
<td>-1.68</td>
<td>1.23</td>
<td>0.21</td>
</tr>
<tr>
<td>Static Compliance</td>
<td>2.82</td>
<td>15.75</td>
<td>28.68</td>
<td>0.02</td>
</tr>
<tr>
<td>Dynamic Compliance</td>
<td>1.17</td>
<td>8.65</td>
<td>16.13</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 15 Lung Physiology Measured During EVLP
Estimated A1AT treatment effects, as well as a p value for the regression coefficient. At a significance level of 0.05, the treatment had an effect on, ΔPo2/Fio2, Static Compliance and Dynamic Compliance. Peak airway pressure didn’t show any significant difference between treatment and control groups.

**Figure 20 Effect of A1At in lung function**

At a significance level of 0.05 (p value for the regression coefficient), treatment had an effect in lung function (delta P02; static and dynamic compliance). On the left, mean of all cases together and on the right each case. A linear mixed model was used for analysis.
The A1AT group showed significantly lower pulmonary vascular resistance (PVR) in the treatment group. We also saw lower pulmonary artery pressure (PAP) during the time the lung was on EVLP in the treated group. (See Figure 21)

![Figure 21](image)

**Figure 21 Effect of A1At in Pulmonary Vascular Resistance (PVR) and Pulmonary Arterial Pressure**

A1AT treatment had an effect in pulmonary vascular resistance ($p< 0.01$ and pulmonary arterial pressure during EVLP. Mann Whitney was used for analysis.

5.4.4. Permeability Studies

**Perfusate Loss during EVLP**

The perfusate lost during EVLP was significantly lower in the treatment group ($p= 0.01$) (Figure 22)
Figure 22 Effect of A1At in Perfusate Loss

Treatment group lost significantly less perfusate compared with control group estimated $= -84.4374$ (intervals -142.2045 to -26.6702158268) $p=0.011$

Wet/Dry Ratio

The wet to dry (W/D) ratio was measured before EVLP and at the end of EVLP. The pre EVLP ratio was not significantly different in between the 2 groups (median 4.88 vs 4.85; $p>0.999$).

However the W/D ratio from the biopsies taken at the end of EVLP was significantly lower in the A1AT treated lungs (6.534 vs 5.195 $p= 0.038$). Wet to dry ratio at the end of EVLP minus beginning of EVLP (change from baseline) was significantly lower in the treatment group. (Figure 23)
Wet/Dry Ratio calculated with samples taken after EVLP was significantly lower in the A1AT treatment group. Wet/Dry Ratio calculated with samples taken before EVLP (baseline) was not significantly different between the treatment and control groups. Wet/Dry Ratio after EVLP subtracted from the Wet/Dry Ratio that was measured before starting EVLP (change from baseline) was significantly lower in the treated lungs.

**Tight Junction**

ZO-1 expression was increased in the lung tissue from injured donor lungs treated with A1AT. (Figure 24)
Figure 24 ZO-1 Expression
Tight junction integrity assessment by Zonula occludens protein-1 (ZO-1) analyzed with Immunofluorescent staining. A ZO-1 immunohistochemical analysis before and after A1AT treatment. The treated group showed increased ZO-1 expression at the end of EVLP (n = 8; P = 0.05) (five slides per individual). B Representative image of ZO-1 stained tissues at the end of EVLP (both tissues from the same donor). Treatment group showed stronger ZO-1 expression (red staining) compared with control. C. Representative image of ZO-1 expression in a normal lung (not subjected to CIT or EVLP).

5.4.5. Effect of A1AT on inflammation measured by cytokine expression

Multiplex

Cytokines measured in perfusate samples taken 1 hour after the beginning of EVLP and then at 3, 6, 9 and at 12 h during perfusion. Overall pro-inflammatory cytokines trend to be lower in the treatment group. Matrix metalloproteinase (MMP3), chemokine C-C motif ligand 2 (CCL2) and Endothelin-1 (ET-1) were significantly lower in the perfusate from lungs treated with A1AT. Data are summarized in Table 16.

Table 16 Cytokines measured in perfusate

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>MEAN DIFFERENCES</th>
<th>PERMUTATION P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>-18153.16</td>
<td>0.14</td>
</tr>
<tr>
<td>TNF alpha</td>
<td>-4.75</td>
<td>0.37</td>
</tr>
<tr>
<td>IL-6</td>
<td>-619.31</td>
<td>0.16</td>
</tr>
<tr>
<td>MMP3</td>
<td>-667.47</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.25</td>
<td>0.96</td>
</tr>
<tr>
<td>CCL2</td>
<td>-5969.19</td>
<td>0.05</td>
</tr>
<tr>
<td>VEGF</td>
<td>-0.23</td>
<td>0.99</td>
</tr>
<tr>
<td>IL-1 beta</td>
<td>25.21</td>
<td>0.88</td>
</tr>
<tr>
<td>IFN gamma</td>
<td>-0.09</td>
<td>0.95</td>
</tr>
<tr>
<td>IL-1 ra</td>
<td>1418.21</td>
<td>0.30</td>
</tr>
<tr>
<td>IL-1 alpha</td>
<td>-1.44</td>
<td>0.62</td>
</tr>
</tbody>
</table>
Cytokines measured in perfusate. Table with the mean difference and a permutation p-value. A1AT treatment had an effect on endothelin 1, MMP3 and CCL2. In column 2 is the mean difference seen between the treated and untreated lungs. P-value is reported in the third column.

Soluble adhesion molecules measured in perfusate at the same timepoints (1 h, 3 h, 6 h, 9 h and end of EVLP) than cytokines, were lower in the treatment group compared with the controls without achieving statistical significance.

Table 17 Adhesion molecules measured in perfusate

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>MEAN DIFFERENCES</th>
<th>PERMUTATION P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-CAM1</td>
<td>-7.84</td>
<td>0.16</td>
</tr>
<tr>
<td>V-CAM</td>
<td>-1958.11</td>
<td>0.09</td>
</tr>
<tr>
<td>E-selectin</td>
<td>-360.22</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table with the mean difference and a permutation p-value. The levels in the treatment group were overall lower, but the differences between treatment and control didn’t reach significance.

The plots for each cytokines per individual are shown in figure 25.
Plots of cytokines per individual. Matrix metalloproteinase-3 (MMP-3), chemokine (C-C motif) ligand 2 (CCL2), also referred to as monocyte chemoattractant protein 1 (MCP1) and endothelin-1 (ET1) also known as preproendothelin-1 (PPET1), showed significantly lower levels compared with the control group, the differences between the treated and untreated lungs are noticeable.
in the plots. For ET1 MMP-3 and CCL2 the means and standard deviation of all cases are showed on the left side and each individual case on the right. IL-8 and IL-6 are shown per each case.

5.5. Results of the Experiments done in Endothelial Cell Culture

5.5.1. A1AT alleviates ischemia-reperfusion (IR) induced injury by reducing Endothelin-1 expression levels in endothelial cells

The first ET-1 expression was measured after leaving the endothelial cells for 18 h in normal cell culture conditions in the presence of media, the ET-1 expression was 115.52 pg/ml (SD 0.33); after 6 h of CIT, replicated by washing the cells and replacing the media with cold Perfadex® the levels of ET-1 were 0.044 pg/ml (+/- 0.390); the cells were then changed to normothermic Steen® solution to resemble reperfusion during EVLP. After 2 h of reperfusion in Steen®, ET-1 levels were higher in the control group 43.11 pg/ml compared with 38.24 (+/- 0) from the 10 mg/ml treatment group, and 41.94 (+/- 1.8) from de 5 mg/ml treated cells and 43.13 (3.44 +/- 3.44) from the cells treated with 1 mg/ml; results were not significantly different (p > 0.05). After 4 h of reperfusion, ET-1 was 60.78 (+/- 2.54) in the control and 49.57 (+/- 1.25) in the 10 mg/ml of A1AT treatment (p < 0.05) the differences where not significant in the 1 mg/ml (58.95 +/- 1.53) and 5 mg/ml A1AT groups (61.00 +/- 2.55) (p > 0.05). After 18 h of reperfusion, the control group showed the highest ET-1 expression with 115.21 (+/- 0.23) compared with the 10 mg/ml treatment 91.18 (+/- 4.20) (p < 0.0001), the differences were also significant when control was compared with 5 mg/ml where ET-1 was 61.00 mg/ml (+/- 2.55) (p <0.001). Results are shown in figure 26.
A. **ET-1 Expression After IR Injury**

![Bar chart showing ET-1 expression after IR injury and different A1AT doses.](image)

B. **ET-1 Expression After IR Injury and 2Hr Reperfusion**

![Bar chart showing ET-1 expression after IR injury and 2Hr reperfusion with different A1AT doses.](image)
C. ET-1 Expression After IR Injury and 4Hr Reperfusion

D. ET-1 Expression After IR Injury and 18Hr Reperfusion
E. ET-1 Expression After IR Injury and A1AT Treatment

![Graph showing ET-1 expression levels with significance markers](image)

Significance (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** p < 0.0001).

*Figure 26 ET-1 Expression in Endothelial Cell Culture Experiments*

A. **Concentration of ET-1 in mg/ml in the control group.** In green expression of ET-1 in normal cell culture conditions in the presence of media for 18 h. Very low level of ET-1 in Perfadex upon 6 h of CIT indicative of slowed down process of protein expression and synthesis. In orange ET-1 levels in Steen® after 2 hours of reperfusion, red shows ET-1 levels after 4 hours of reperfusion in Steen® and pink ET-1 levels after 18 hours of reperfusion in Steen®. ET-1 levels were significantly different in all groups (p < 0.0001).

B. **ET-1 after CIT and 2 h reperfusion.** Comparison of ET-1 levels in control group and different doses of A1AT (1mg/ml, 5 mg/ml and 10 mg/ml) didn’t show any statistical differences after CIT and 2 h of reperfusion with normothermic Steen® (p > 0.05).

C. **ET-1 after CIT and 4 h reperfusion.** ET-1 levels were significantly lower in the cells treated with 10 mg/ml of A1AT compared with the untreated cells (p < 0.05).

D. **ET-1 after CIT and 18 h of reperfusion.** ET-1 expression were significantly lower (p < 0.001 in cells treated with 5 mg/ml A1AT, and p < 0.0001 in cells treated with 10 mg/ml), ET-1 from cells treated with 1 mg/ml were not significantly different than control.

E. **Summary of ET-1 Expression in cells treated with 10 mg/ml A1AT.** In green expression of ET-1 in normal cell culture conditions in the presence of media for 18 h (control). In orange ET-1 levels in Steen® after 2 hours of reperfusion, red shows ET-1 levels after 4 hours of reperfusion in Steen® and pink ET-1
levels after 18 h of reperfusion in Steen®. ET-1 levels were significantly lower at all timepoints in comparison with the ET-1 expression after 18 h in regular media (p < 0.0001).

5.6. Discussion

In recent studies, A1AT has shown to have anti-inflammatory properties. We assessed the effect of A1AT in donor lung function, inflammation and endothelial function in human lungs that were unsuitable for transplantation. In this study, the drug was delivered intravascularly by adding A1AT into the circulating perfusate during EVLP. Using 10 mg/ml of A1AT we were able to achieve stable levels of A1AT during the 12 h of perfusion. NE-A1AT complex was significantly higher in the treated group confirming that exogenous A1AT is able to bind NE.

As an indirect measure of increase permeability, A1AT-treated lungs lost significant less perfusate during the course of the EVLP compared to control lungs, supporting our hypothesis that endothelial cell function is protected by A1AT. The effect of A1AT on the lung endothelium was further confirmed when we stained lung tissue to assess the endothelial tight junctions. Lungs that received A1AT during EVLP had significantly higher tight junction protein ZO-1 expression (p=0.03). The protective effect of A1AT on the endothelial function was likely mediated by reduced expression of ET-1. ET-1 is known to mediate acute lung injury. Overexpression of ET-1 leads to endothelial dysfunction and nitric oxide production that induces primary graft dysfunction.\cite{Machuca et al. 2015} Inhibition of ET-1 seems to have a protective effect early after transplantation. A1AT also reduced expression of other pro-inflammatory markers related to endothelial cell dysfunction such as CCL2 and MMP3. Increased expression of matrix metalloproteinases (MMPs) has been observed in almost every disease involving inflammation. MMPs are known to regulate physical barriers and control inflammatory mediators (cytokines and chemokines) as well as create chemokine gradients in tissues that are injured. Excessive expression of MMP correlates with the
pathogenesis of acute lung injury, the exact mechanisms hasn’t been identified but it is known to interrupt junction proteins in endothelium. CCL2 expression correlates with monocyte recruitment and inflammation as well as vascular activation. CCL2 in the endothelium produces cell retraction and vascular leakiness.

The main advantages of using human lungs are the use of a human derived drug to human patients, avoiding the possible difference in the species confounder factor, as well as the exemplification of the “real life” complex lung injury setting. We decided to test A1AT in one lung using the contralateral as control. For this tactic, we divided the lung block and tested the effect of the drug on one of the lungs (randomly assigned to receive treatment) and compared the results with its paired lung. We also compared all treated lungs with the untreated ones. Being able to test the effect of the drug in a lung compared to another lung that suffered similar insults (both coming from the same donor), gives us the unique opportunity to appreciate the results in a comparable and controlled setting.

This approach, beneficial in many ways, adds a complexity factor when analyzing the results. The use of standard methods of logistics like regression modeling would analyze the data without taking the association between the 2 lungs of the same donor into account. Lungs that come from the same donor may also have different types of injury, the worse or the best side could be chosen to be the treated one. However, in our study the lungs were randomly selected to be control or treatment. For the physiological data (delta pO₂, compliance, peak airway pressure) we decided to use a linear mixed model for the analysis.

An additional advantage to this approach is that standard analysis software and techniques can be used. The disadvantage is that not all side specific data are used. Separate analyses could be carried out on each side to avoid this but spreading the data over two analyses would mean loss of
statistical power — therefore reducing the chances of the study finding significant results — when associations do exist. There is also the added problem of the interpretation (the two analyses clearly are not independent), especially if the results for each side differ.

One of the limitations of this study is the small sample size and the need for adjustment for for multiple comparisons. Furthermore the inflammatory markers studied are not independent variables, but are in fact biologically related. After the initial experiments there was an a priori expectation that some of the cytokines related to endothelial cell function (ET-1) could be affected by A1AT. After Bonferroni correction the cytokine differences did not achieve significance. A Bonferroni threshold for the 16 perfusate tests would be 0.05/16 = 0.003, but, as noted above, this level of significance is not achievable with the small sample size available. This was one of reasons why we decided to focus on the specific effect of A1AT in Endothelin-1 in the endothelial cell model.

After demonstrating the effect of A1AT at protecting the lung endothelium we decided to further investigate the mechanistic effects by which A1AT was protecting the endothelial cell function. For this, we used a human endothelial cell culture ischemia-reperfusion model and tested the specific effect of A1AT given during normothermic EVLP. A1AT treated cells showed lower levels of ET-1 in a dose and time dependent manner.

EVLP has shown to be an excellent platform to test new potential therapeutics, in this study we were able to see the effects of the drug in real time in terms of improvement of the lung physiology. With the EVLP perfusate and lung tissue that we collected we were able to test its potential effect on pro-inflammatory cytokines and permeability. We saw a beneficial effect in terms of protection of the lung endothelium, A1AT inhibited ET-1 expression and improved the ZO-1 junctions. The endothelial cell culture experiments demonstrated the effect of A1AT at inhibiting ET-1 as one of
the mechanistic effects of the endothelium protection also showed in the human lung experiments. The endothelial permeability could also be tested with the electric cell substrate impedance system (ECIS) platform as a future direction for this study. Additional experiments can be performed to evaluate if A1AT is protecting the endothelial barrier by measuring other measurements of endothelial injury measured, such as angiopoietin-2, von Willebrand factor, or thrombomodulin. Markers for lung epithelial injury such as RAGE or SP-D or cytokeratin-18 may also be measured. One of the positive finding during this experiments was the reduction of the pulmonary vascular resistance (PVR). PVR is known to be elevated in patients presenting acute respiratory distress syndrome.\(^{(M\text{atthay and Zemans 2011)}}\) Disturbance of the endothelium may result in a disruption in the normal balance of vasodilators like NO, prostacyclin, and vasoconstrictors including thromboxane, leukotrienes, endothelin, serotonin, and angiotensin II, causing vasoconstriction and finally increasing PVR.\(^{(B\text{ull et al. 2010; Ryan, Frohlich, and McLoughlin 2014)}}\) The decrease in PVR evidenced in the A1AT treated lungs could be translating into less pulmonary edema. The exact mechanism by which A1AT could be reducing the vascular pressure should be further analyzed in future studies. One of the potential ways by which A1AT could be reducing pulmonary edema was by lowering the pulmonary arterial pressure (PAP). It has been demonstrated that an increase in pulmonary artery pressure may produce an increase in extravascular lung water, probably connected to changes in pulmonary capillary pressures. We documented PAP of the lungs during 12 h of EVLP and, although we saw a decrease in the PAP in the treated group (specially towards the later time points) in comparison with the controls, we didn’t find evidence of such a high PAP correlating with the lung injury that would explain the increase in lung edema in the untreated lungs.
We saw some reduction of the pro-inflammatory cytokines but we didn’t reach a statistically significant decrease that we were expecting and that we saw in previous studies. One of the main reasons could be that in this setting, EVLP resembles the ischemia-reperfusion scenery, but we are still missing the alloimmune response that comes after transplant. The effect of A1AT given during EVLP in modulating the alloimmune response after transplant will be answered in a clinical trial. In conclusion A1AT seems to have a positive effect in lung function by protecting the lung endothelium and inhibiting ET-1. We believe that the use of A1AT during EVLP may prevent the development of primary graft dysfunction by protecting the lung endothelial function.
Chapter 6. Summary and Future Directions
6.1. Summary

The first successful lung transplant was performed in Toronto by Cooper et al in 1983 and lung transplantation is now established as the only curative treatment for many patients suffering from end-stage lung diseases. With improvements in medical and surgical care, the results of lung transplantation have improved and the number of lung transplants performed each year has increased exponentially as this life-saving treatment can be offered to more and more people. The demand for lung transplant has led to an increasing shortage of donor lungs. Many programs have started accepting extended criteria donor lungs to alleviate the donor shortage but even the most aggressive transplant centers are able to utilize only 20-40% of potential donor lungs. The main concern when using these suboptimal donor lungs is the fear that the recipient may have a higher risk of developing primary graft dysfunction (PGD) after transplantation. Severe PGD not only the most significant cause of early mortality and morbidity but is also associated with worse long term outcomes after lung transplantation. There is currently no agent approved to prevent or treat PGD, essentially supportive care is the only alternative that we have to treat patients with severe PGD in the intensive care unit. Novel therapies to mitigate the effects of PGD would not only improve post-transplant outcomes but could also increase the donor pool by allowing further extension of criteria to accept a donor lung.

The most significant risk factor for PGD is ischemia reperfusion injury (IRI). Many agents have been studied experimentally to reduce IRI and PGD, but there is a lack of translation of basic science discoveries to clinical practice. Many treatments that have been promising in cell culture and rodents have failed to show benefit in clinical trials. In order to examine potential therapeutics to avoid PGD, there is a clear need for a translational pathway to test novel basic science discoveries in relevant pre-clinical models to choose the most promising approaches for clinical
trials. We studied the use of A1AT in preventing PGD using novel translational large animal and ex-vivo lung perfusion models to allow for planning a human clinical trial.

The first part of the thesis focused on the development of a pig single lung transplant survival model to test potential therapeutics and promote pre-clinical trials for PGD treatment. During the development of the model we not only focused on the surgical steps, we also had to adjust the post-transplant medical management to optimize outcomes. Adjusting antibiotics, and antithrombotic prophylaxis. One of the more challenging parts was to achieve adequate pain management without any side effects.

Our group has investigated the effects of A1AT in cell culture and rat models as well as non-survival pig transplant model, suggesting a potential benefit of A1AT in reducing IRI. In the second part of the study, we use the pig single lung transplant model to determine dosing regimen, safety and efficacy of A1AT. We identified that A1AT is rapidly consumed in the pro-inflammatory setting of IRI lung injury and recognized that daily administration of A1AT is required. We showed that A1AT is safe, well tolerated and clinically beneficial in lung transplantation, inducing faster recovery and less oxygen need after transplant. We did not observe similar changes in lung function or inflammatory markers seen in our prior studies, likely explained by the fact that all the pigs had fully recovered from the early injury by 3 days. However, the accelerated recovery seen in A1AT-treated pigs supported a biologically important role for A1AT in preventing IRI.

The third part of the thesis focuses on A1AT treatment of clinically rejected human lungs during EVLP. EVLP is the ideal platform to test new therapeutics as it allows real-time measurement of lung function in human lungs without posing patients to any risk. By randomizing one lung to receive A1AT and the other lung as a control, we had the unique opportunity of not only comparing
the results between the treatment and control groups, but also in between lungs that came from the same donor, reducing the “noise” that comes from comparing organs from different individuals. Our hypothesis was that A1AT has anti-inflammatory effects in the donor lung that lead to improved endothelial cell function. During intense inflammation such as seen in IRI, the endothelial cell to cell junctions are disrupted resulting in increased fluid leakage into the air space and thereby worsened gas exchange and lung compliance.

Using severely injured lungs, we were able to show significant better function of A1AT-treated lungs. A1AT-treated lungs lost less perfusate during the course of the 12 h EVLP compared to control lungs, supporting our hypothesis that endothelial cell barrier function is improved by A1AT. This protective effect of A1AT was further confirmed when we stained lung tissue to assess the endothelial tight junctions. Lungs that received A1AT during EVLP had significantly higher tight junction protein ZO-1 expression. The protective effect of A1AT on the endothelial function was likely mediated by reduced expression of ET-1 that has been shown to induce PGD by disrupting the alveolar-capillary membrane. A1AT also reduced expression of other pro-inflammatory markers related to endothelial cell dysfunction such as CCL2 and MMP3 that both have been shown to promote vascular leakiness.

The effect of A1AT in the endothelium was explored specifically in the context of human lungs that were unsuitable for transplantation. A1AT seems to be acting by enhancing cell junctions and decreasing various pro-inflammatory cytokines related to endothelial function but the exact mechanism still remains unknown. Using an endothelial ischemia reperfusion in vitro model we could assess other specific mechanistic effects in depth, including the validation of the hypothesis about A1AT protecting the lung endothelium by reducing ET-1.
A1AT could also be acting by reducing vascular resistance (evidenced by PVR decrease in the treatment group). The exact mechanism by which A1AT may be reducing the lung endothelial and potentially the lung epithelial lung barrier should be further investigated.

In this study, we bridged the gap between our promising results of A1AT in *in vitro* and small animals studies and the clinical setting by using two promising and clinically relevant pre-clinical models, the pig single lung transplant model and EVLP-treated rejected human donor lungs. We showed that A1AT was consistently effective in preventing IRI in these models that are as close to the clinical setting we can get without a true clinical trial. However, we also learned that some of the mechanisms of action were different between our rodent work and the EVLP-model and that the dosing used in our small animal models and pig lung transplant survival model were vastly different. This translational approach is well-suited to support drug development in a step-wise fashion Figure 27.

*Figure 27 Drug development pipeline using a translational medicine approach*
In summary, starting with basic science research done in epithelial cell cultures, rat ischemia reperfusion injury and a non-survival pig single lung transplant, we have shown A1AT to be a promising agent for the treatment of PGD (Figure 28). In this study, we have used directly relevant new models to validate our basic science findings and test the appropriate dosing on large animal and human EVLP-platforms. Based on our findings, we feel confident to move forward with a randomized prospective clinical trial to use A1AT to prevent PGD in the hopes of bringing the lung transplant community the first effective therapy for PGD. This clinical trial will be initiated within the next year in the Toronto Lung Transplant Program.

![Figure 28 Experiments performed in our lab to test the effect of A1AT in reducing ischemia reperfusion lung injury](image)

The experiments were done in escalating complexity starting with cell culture models, small animal, large animals and finally human lungs using ex vivo lung perfusion.

### 6.2. Future Directions

#### 6.2.1. Clinical Trial of A1AT during EVLP before transplantation
Rationale and hypothesis of the study

EVLP has shown to be an ideal therapeutic platform to increase donor utilization and improve postoperative outcomes. The Toronto Lung Transplant Program currently transplants 70% of donor lungs that are assessed on EVLP and, using this technology, has successfully doubled the overall lung transplant volumes from 100 to 200 lung transplants annually—currently 40% of these transplanted lungs are treated with EVLP.

In other programs, the fraction of EVLP-treated donor lungs going forward to lung transplant is much lower and ranges in the 10-30% range and many centers have yet to adopt EVLP until a solution to this problem is found.

As we explained throughout the thesis, we now have very strong data supporting the use of A1AT to prevent PGD after lung transplantation and a good understanding of A1AT dosing during EVLP. A1AT also has an excellent safety profile, further supporting a clinical trial in lung transplantation. We hypothesize that A1AT treatment during donor lung EVLP will:

Increase the utilization rate of donor lungs treated with EVLP; and

Decrease the length of stay in the intensive care unit (ICU) after lung transplantation

We believe that clinical evidence of A1AT during EVLP therapy that leads to rehabilitated lungs with less post-transplant complications would result in an increase in the utilization rate of lungs in centers using EVLP, encourage widespread adoption of EVLP in lung transplant centers, and improve lung transplant outcomes worldwide.

**Study plan**

The next step would be a randomized, controlled double-blinded single center clinical trial to evaluate the efficacy of A1AT in EVLP-perfusate in donor lungs treated with EVLP.

**Inclusion criteria**
**Donor lungs:** We will include all donor lungs retrieved by our surgical team that undergo EVLP-treatment prior to lung transplant due to not meeting criteria for direct lung transplantation. High-risk donor lungs eligible for EVLP are defined as those meeting any one of the following criteria:

- Best ratio of the partial pressure of arterial oxygen (PaO2) to FIO2 of < 300 mm Hg;
- Pulmonary edema, defined as bilateral interstitial infiltrates without evidence of infection, detected on the last chest X-ray image by the lung transplant physician assessing the donor;
- Poor lung deflation or inflation during direct intraoperative visual examination at the donor site;
- Other concerning donor risk factors such as blood transfusions exceeding 10 units;
- A deceased after cardiac death donor where the transplant surgeon has concerns on donor lung function

**Patients:** We would include adult patients listed for double-lung transplantation in Toronto General Hospital. Potential study participants would be consented for the study at the time of lung transplant listing.

**Exclusion criteria**

**Donor lungs:** We will exclude all donor lungs that proceed directly to lung transplant. We will also exclude EVLP-treated donor lungs that are hepatitis C positive or that undergo EVLP due to logistical reasons as the indication for EVLP is not related to lung injury.

**Recipients:** Patients bridged to lung transplant with mechanical ventilation or extracorporeal life support. Pediatric recipients (<18 years of age) will also be excluded.

**Study design**

When a potential donor lung meeting the inclusion criteria is available for a study patient, the lung will be placed on EVLP and randomized 1:1 to control/A1AT arms. The transplant team and organ procurement organization (Trillium Gift of Life Network) will be blinded in terms of treatment
group. A1AT/control will be administered in the perfusate (10 mg/mL) throughout the experiment. We have tested this dose in our preliminary studies and shown it to be effective and result in stable perfusate A1AT-concentrations of 4-5 mg/mL as A1AT is both taken up by the lung tissue as well as consumed during the process. Physiological and biological testing will be performed on the lungs while on EVLP. After 4-6 hours of EVLP, the transplant surgeon will decide if the donor lungs meet transplant criteria (First primary outcome). The recipients will receive conventional treatments according to our clinical protocols both peri- and postoperatively. The second primary outcome is the length of ICU stay in days and this will be specifically captured by the study coordinator. This will be determined by using the arrival to ICU as the start time and the end time will be the time when the ICU attending signed the transfer order to discharge the patient from the ICU.

The primary preoperative end-point will be safety of the intervention. Secondary outcomes will include the utilization rate of donor lungs (i.e., percentage of donor lungs recruited to the study advancing to lung transplant) between A1AT-treated and control donor lungs changes in oxygenation, lung compliance, airway pressures, pulmonary vascular resistance and loss of perfusate (Steen® solution) during 4-6 hours of EVLP. The secondary postoperative end point will be length of stay in the ICU. As the main reason for prolonged ICU stay is failure to wean off mechanical ventilation, this is an appropriate marker of early lung allograft dysfunction. In our experience, the International Society for Heart and Lung Transplantation (ISHLT) definition of PGD is a relatively poorer measure of early lung allograft injury compared to ICU length of stay and, thus, will only be used as a secondary outcome measure. Also, measuring the time a patient spends in the ICU will allow for better modeling of cost-effectiveness, as improvements to this metric will significantly reduce overall lung transplant costs.
Secondary outcomes will include time to extubation, time to weaning off supplemental oxygen, ISHLT PGD grade at 0, 24, 48, and 72 hours, length of hospital stay, and 30-day mortality.

In order to fully understand the therapeutic effects of A1AT during EVLP, we will also include biological testing. The following studies will be conducted: 1) Inflammatory mediators: IL-1B, IL-6, IL-8, IL-10, TNFα, NFKβ, MMP3, and CCL2. 2) Cell death signal: M30, M65, HMGB1. 3) Endothelial function: ET-1, big ET-1, NOx, sICAM1 and sVCAM1. We have previous demonstrated that these measurements are valuable biomarkers to predict clinical EVLP lung performance. In addition, we would like to conduct metabolomics and proteomics studies on the perfusate to screen for new biomarkers and investigate mechanisms of PGD.

Randomization and blinding will be done using appropriate software, with additional assistance from CSL Behring to randomize drug supply in a blinded fashion.

Study power (Beta=0.2, alpha=0.05)

We currently use EVLP in ~ 50% of donor lungs that we retrieve for lung transplant. Of the donor lungs that undergo EVLP, we currently use 70% for lung transplant while 30% are rejected from lung transplant. We hypothesize that A1AT treatment will increase the utilization rate from 70% to 85-90%. Furthermore, around 32% of patients have an ICU length of stay (LOS) shorter than three days, and we hypothesize that in treated lungs that are transplanted, 50% will have ICU LOS shorter than three days.

The composite outcome is defined as a transplant followed by an ICU LOS in the recipient shorter than three days. We hypothesize that the proportion of lungs with this composite outcome will increase from 0.7 X 0.32 = 0.224 in the control group to at least 0.85 X 0.50 = 0.425 in the treated group. Under these assumptions, 84 lungs per group gives 80% power at a type I error rate of 5% in a comparison of the difference of proportions with the composite outcome. We currently receive
110-120 donor lungs for EVLP on an annual basis; so, we would need to recruit for approximately 24 months for a fully powered study.

Each of the components of the composite will be examined as secondary outcomes. With 84 donor lungs per group, we have 80% power for an increase from the current value of 70% to 88% in the percentage of lungs used. In the expected numbers of lungs proceeding to transplant (70% of 84 and 85% of 84), we have 80% power to show an in improvement from the current value of 32% to a value of 56% in the percentages of patients with ICU LOS less than three days. Our current median ICU length of stay after bilateral lung transplantation is 4.6 days (Interquartile-range 2.5-12.7). One third (32%) of patients have an ICU-stay of <3 days. We specifically hypothesize that A1AT therapy will increase the proportion of patients leaving the ICU in <3 days from 32% to 50%. The ICU stay is affected by many factors and is not solely dependent on the quality of the donor lungs. We believe that the 3-day time point will best capture the patient group most likely to benefit from A1AT-therapy.

As a fully-powered study is a major undertaking, we propose to start with a preliminary study of 20 patients in each group (total n=40). We propose to have an independent external supervisory committee appointed to assess safety and efficacy of the A1AT-treatment after the preliminary phase.

**Study timeline**

A fully powered study to show both an increase in donor lung utilization rates as well as decreased ICU length of stay will require 164 donor lungs and lung transplant recipients recruited into the study. With our current volumes, this would be achieved in around 1.5-2 years. As stated above, we propose to perform an interim analysis when 40 patients (20 A1AT + 20 controls) have been transplanted. Recruitment of 40 patients is estimated to take 6-9 months and given the early time
points, we should have an answer to the composite outcome in 9-12 months after study initiation. We generally have experienced a very high patient consent rate in our clinical studies so we do not foresee patient recruitment as a major issue.

**Cost-effectiveness of A1AT+EVLP-therapy**

It is expected that for each additional lung transplant performed, the economic impact of the increase in Quality Adjusted Life Years (QALY) is $606,869/patient ($69,755 x 8.7 added years), in addition to wait list time savings of $21,400/patient giving a total of $628,000/ lung transplant. If we can increase the rate of lung utilization from 70% to 85-90%, we will increase the number of lung transplants by 20-25/year resulting in an annual cost benefit of $12-15 million.

Furthermore, the current cost for a lung transplant in Canada is $150,000. The majority of the costs are related to the operation itself as well as the ICU-care after the operation. If we can shorten the ICU-stay by an average of 1-2 days/patient at the average cost of $3,000 per ICU day, we expect further savings of $3,000-6,000 per patient.

**Significance of the study**

If our hypothesis holds true, this will be a first in man study to show efficacy of pre-transplant donor therapy in treating EVLP donor lungs that result in decreased early lung allograft injury. Currently, no such therapy exists. Positive outcomes will change the role of EVLP from an assessment tool to a treatment modality and not only increase the utilization rate of donor lungs, but also improve early postoperative outcomes. Given our significant increases in donor lung utilization at UHN due to EVLP use, we envision the worldwide adoption of EVLP to safely assess and rehabilitate donor lungs for the majority of lung transplant cases moving forward, thus creating a platform for the widespread integration of A1AT therapy as part of the future standard donor lung transplant regimen. Importantly, our study outcomes will also provide rationale to test A1AT
recipient therapy without EVLP in a subsequent study/trial, and provide a future foundation for A1AT testing in additional ex vivo organ transplant modalities.
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Chapter 8. Appendix Contributions
Lindsay Caldarone assisted with study design, surgical assistance, sample collection, performing the assays, analyzing the data for the pig and the first part of the human studies. Dr. Antti Nykanen helped with the experiments regarding the treatment with A1AT for human lungs during EVLP, study and reviewed the manuscript. Dr. Marcos Galasso and Aadil Ali assisted during the EVLP experiments. Allen Duong helped with sample collection and processing of the human and pig samples. Dr. Andrew Sage contributed to the human studies cytokine analysis.

Xiaohui Bai and Anette Gower assisted with the ELISA experiments, Guan Zehong assisted with the ZO-1 staining. The statistical analysis for the human rejected lungs treated during EVLP were performed by Julie Hudson from Biostatistics Research Unit (BRU), Ricardo Zamel gave advice regarding biostatistics.

Dr. Wenxi Gao led the cell culture and rat ischemia reperfusion studies, Dr. Ilker Iskender led the study assessing A1AT in a non-survival pig lung transplant model. Dr. Huiqing Lin led the study assessing A1AT in pig lungs in EVLP. Sahar Soltanieh performed the endothelial cells in vitro studies.

Dr. Shaf Keshavjee supervised, supported, and provided feedback for the thesis. Drs. Mingyao Liu and Marcelo Cypel were PAC committee members and provided invaluable feedback and guidance throughout the project. Dr. Jussi Tikkanen wrote part of the proposal for the potential clinical trial, reviewed the manuscripts and gave instrumental advice throughout the project. Drs. Tereza Martinu, and Stephen Juvet, provided feedback, guidance, and suggestions throughout the project.