

Ex Vivo Lung Perfusion

- Experimental and Clinical Studies

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Ex Vivo Lung Perfusion – Experimental and Clinical Studies

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ABSTRACT

Background: Ex vivo lung perfusion (EVLV) has since its introduction in clinical practice experienced a rapid expansion and made more organs available for transplantation. Different protocols and strategies have been implemented at transplantation centres around the world.

Aims: In an experimental setup in pigs, the effect of haemofiltration during EVLP on lung function, perfusate oncotic pressure and lung weight (paper I), was evaluated, and two clinically used strategies for EVLP were compared, with respect to lung function, metabolism, inflammatory response, oxidative stress, and cell viability (paper II). To assess the clinical outcome of patients in Gothenburg and Copenhagen undergoing lung transplantation after EVLP they were compared to a contemporary control group (paper III). Correlations between lung physiologic variables during EVLP and short-term clinical outcome in lung transplant recipients were assessed, with the intention to identify variables during EVLP predicting post-transplantation outcome.

Methods: In paper I, pulmonary oedema was induced in pigs, and lungs randomized to EVLP with or without haemofiltration. Oncotic pressure, lung performance and weight were measured before and after EVLP. In paper II porcine lungs were harvested and randomized to EVLP according to either of two clinically used protocols. The groups were compared before and after four hours of EVLP. In paper III lungs not accepted for donation, but with potential for improvement, underwent EVLP and were transplanted if predefined criteria were met. Outcome was compared to a control group of patients transplanted with conventional donor lungs. Variables during EVLP were examined for correlation with short-term outcome after lung transplantation in paper IV.

Results: Haemofiltration during EVLP increased oncotic pressure and decreased lung weight compared to EVLP without haemofiltration, but without effect on lung oxygenation capacity in either group (paper I). There was a trend towards more lung oedema formation in the acellular, open left atrium group, but otherwise there were no differences between groups (paper II). Patients receiving lungs after EVLP had a lower PaO₂/FiO₂ ratio at arrival in the intensive care unit (ICU), longer time to extubation and spent longer time in ICU, however without difference in lung function at one year or survival at intermediate follow-up (paper III). No correlations could be found between variables measured during clinical EVLP and short-term outcome in lung transplant recipients (paper IV).

Conclusions: Haemofiltration during EVLP may decrease pulmonary oedema. No major differences in effect could be established between the two clinically most used methods for EVLP. Outcome in patients transplanted with lungs after EVLP was comparable to patients receiving conventional lungs at intermediate-term follow-up. There were no clear correlations between commonly measured variables during EVLP and short-term outcome.

Keywords:

Ex vivo lung perfusion, EVLP, lung transplantation

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

- I. T. Nilsson, C. Hansson, A. Wallinder, C-J Malm, M. Silverborn, S-E Ricksten, G. Dellgren.
Hemofiltration in ex vivo lung perfusion - a study in experimentally induced pulmonary edema.
The Journal of Thoracic and Cardiovascular Surgery. 2016;151(2):570-575.
- II. T. Nilsson, J. F. Gielis, A. Slama, C. Hansson, A. Wallinder, S-E. Ricksten, G. Dellgren.
Comparison of two strategies for ex vivo lung perfusion.
The Journal of Heart and Lung Transplantation. 2018;37(2):292-298.
- III. T. Nilsson, A. Wallinder, I. Henriksen, J. C. Nilsson, S-E. Ricksten, H. Møller-Sørensen, G. Riise, M. Perch, G. Dellgren.
Lung transplantation after ex-vivo lung perfusion in two Scandinavian centres.
Submitted manuscript.
- IV. T. Nilsson, A. Wallinder, I. Henriksen, J. C. Nilsson, S-E. Ricksten, H. Møller-Sørensen, G. Riise, M. Perch, G. Dellgren.
Correlation of factors during ex vivo lung perfusion with short-term outcome post transplantation.
Manuscript.

SUMMARY IN SWEDISH

SAMMANFATTNING PÅ SVENSKA

Lungtransplantation är en etablerad slutgiltig behandling för patienter med terminal andningssvikt av olika genes. Långtidsresultaten är goda, och patienter transplanterade vid Sahlgrenska Universitetssjukhuset har en femårsöverlevnad på omkring 80%, vilket i en internationell jämförelse är mycket bra.

Tillgången på organ är dock en begränsande faktor och trots ett långsiktigt arbete för att öka tillgången på donerade organ, genom bättre urvalsprocesser och användande av marginella organ från fler donatorer, är det fortsatt kö till lungtransplantation. Andelen multiorgandonatorer som donerar lungor varierar stort mellan olika centra, och det föreligger ett behov av att öka andelen organ som kan tas tillvara.

Ex-vivo lungperfusion (EVLP) utvecklades som en metod för att utvärdera lungor utanför kroppen, initialt beskrivet av Stig Steen och kollegor 2001. De beskrev i en studie hur lungor transplanterades från en hjärtdöd donator till en recipient efter att lungorna utvärderats med hjälp av EVLP. EVLP har sedan dess genomgått en snabb utveckling och används nu kliniskt på ett stort antal lungtransplantationscentra världen över. Man kan, när man tror att patologin är reversibel, utvärdera, bedöma och optimera marginella organ, för att därefter ta beslutet att transplantera. Lundprotokollet som perfunderar med blodkroppsinnehållande perfusat (cellulärt perfusat) och öppet vänster förmak, samt evaluerar under fullt flöde, var det första protokollet beskrivet av Stig Steen. Torontoprotokollet som perfunderar med acellulärt perfusat i en sluten krets och evaluerar vid lägre tryck och flöden, beskrevs 2008, och har därefter blivit tongivande. Ytterligare ett protokoll, det s.k. OCS-protokollet, som möjliggör EVLP under transport, har utvecklats.

Utöver möjligheterna att noggrant utvärdera funktionen hos lungorna, erbjuder EVLP vissa terapeutiska möjligheter. Man kan ”lufta upp” sammanfallna lungdelar under ögats överinseende, inspektera (bronkoskopi) och suga rent i luftvägarna, samt genom att perfundera med en särskild, för ändamålet framtagen lösning, med hyperonkotiska egenskaper, reducera vätskeansamling i lungorna (lungödem).

I arbete I visades i en djurexperimentell studie på grislungor, i vilka lungödem framkallades genom att strypa det venösa avflödet från lungorna och ge stora mängder kristalloid vätska samtidigt, att man genom att koppla ett hemofilter till perfusionskretsen under EVLP, och hemofiltrera perfusatet, kunde öka det kolloidosmotiska trycket i perfusatet och därmed öka den ödemreducerande effekten av EVLP.

I arbete II jämfördes, i en djurexperimentell studie, de två förhärskande strategierna för EVLP, Lund- och Torontoprotokollen, så som de kliniskt appliceras i Göteborg respektive Wien. Hälften av lungparen randomiserades till vardera gruppen och genomgick 4 timmar EVLP, enligt respektive protokoll. Organen utvärderades avseende vikt och ödemutveckling, lungfunktion, inflammatoriskt svar, ischemi-reperfusionsskada och histopatologiskt utseende. Några avgörande skillnader mellan metoderna kunde inte påvisas.

I arbete III, studerades utfallet på kort och lång sikt efter transplantation med lungor som genomgått EVLP, hos 54 recipienter, jämfört med en samtida grupp patienter som transplanterats med konventionella lungor. Tid till extubation och tid på IVA var signifikant längre hos patienter som fått lungor som genomgått EVLP, men det var ingen skillnad i tid till utskrivning från sjukhus, lungfunktion efter ett år eller överlevnad utan re-transplantation.

I arbete VI, studerades, som ett led i att bättre förstå vilka faktorer under EVLP som påverkar lungornas funktion i recipienten efter transplantation, korrelationen mellan ett antal parametrar under EVLP med tre utfallsmått efter transplantation, första $\text{PaO}_2/\text{FiO}_2$ vid ankomst till IVA, tid i ventilator och tid på IVA. Vi kunde inte finna några signifikanta korrelationer, och i uni- och multivariat analys fanns inte några samband mellan det vi vanligen mäter under EVLP och korttidsresultaten efter transplantation.

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ABBREVIATIONS

| | |
|---------------------------|--|
| ABG | Arterial blood gases |
| ACA group | Acellular perfusate and closed left atrium, study group in paper II with |
| ARDS | Acute respiratory distress syndrome |
| BOS | Bronchiolitis obliterans syndrome |
| BW | Body weight |
| CLAD | Chronic lung allograft dysfunction |
| CO | Cardiac output |
| COA group | Cellular perfusate and open left atrium, study group in paper II with |
| COPD | Chronic obstructive pulmonary disease |
| DBD | Donation after brain death |
| DCD | Donation after cardiac/circulatory death |
| $\Delta P_{O_2}/F_{iO_2}$ | Transpulmonary oxygen gradient ratio |
| EtCO ₂ | End-tidal carbon dioxide |
| ESR | Electron spin resonance |
| EVLV | Ex vivo lung perfusion |
| FEV ₁ | Forced expiratory volume in the first second |
| FiO ₂ | Inspired fraction of oxygen |
| GAPDH | Glyceraldehyde-3-phosphatedehydrogenase |
| HF | Haemofiltration |
| HF group | Haemofiltration group in paper I |
| HIF-1 α | Hypoxia inducible factor-1 alpha |
| ICU | Intensive care unit |
| IL-x | Interleukin-x |
| ISHLT | International Society for Heart and Lung Transplantation |
| LA | Left atrium |
| LTx | Lung transplantation |
| MAC | Minimal alveolar concentration |
| MAP | Mean arterial pressure |
| mRNA | Messenger ribonucleic acid |
| nonHF group | Non-haemofiltration group in paper I |
| P-F/ratio | PaO ₂ /FiO ₂ |
| PA | Pulmonary artery |
| PAs/PAm/PAd | Systolic/mean/diastolic pulmonary artery pressure |
| PaO ₂ | Pulmonary Arterial Oxygen Tension |
| PCWP | Pulmonary capillary wedge pressure |
| PEEP | Positive end-expiratory pressure |
| PGD | Primary graft dysfunction |
| PIP | Peak inspiratory pressure |
| PO ₂ | Oxygen partial pressure |

| | |
|-----------------|--|
| PVR | Pulmonary vascular resistance |
| PVRI | Pulmonary vascular resistance index |
| Qp | Perfusate flow rate |
| qPCR | Quantitative polymerase chain reaction |
| ROS | Reactive oxygen species |
| SO ₂ | Oxygen saturation |
| TNF- α | Tumour necrosis factor alpha |
| Vt | Tidal volume |

INTRODUCTION

Lung transplantation

In 1963, Hardy and colleagues in the USA, reported the first human lung transplantation, with lungs retrieved from a donor after cardiac death, on a convict with lung cancer and obstruction of the distal airways with recurring pneumonias.^{2,3} This first patient survived 18 days after transplantation, but subsequently died due to renal failure. Over the coming years, few transplantations were performed worldwide, with disappointing outcomes.⁴ In 1970 a review of 23 lung transplantations performed up until then, reported only one patient surviving longer than a month.⁵ In that single case, the patient died after 10 months in what was described as chronic rejection.⁶ Poor healing of the airway anastomosis, secondary to the prevailing immunosuppressive regimes with high doses of prednisolone, impeded anastomotic site healing and resulted in poor survival.⁷

The introduction of cyclosporine to the immunosuppressive arsenal, with significant improvements in survival after kidney and liver transplantation,⁸ triggered new interest into heart-lung and lung transplantation. The advent of cyclosporine enabled steroid doses to be kept considerably lower, and this, together with improved surgical techniques, promoted anastomotic site healing.⁹

Successful combined heart-lung transplantation performed in 1981 indicated that transplanted lungs could function in a recipient.¹⁰ In 1983 the Toronto Lung Transplant group performed the first successful single lung transplantation, in a 58-year-old man with pulmonary fibrosis, still alive at the time of publication of the results in 1986.¹¹ At the same centre, the first double lung transplantation was performed in 1986 in a 46-year-old woman with emphysema. She survived until 2001, for almost 15 years, and succumbed to unrelated illness.¹²

These events laid the foundation for a rapid development and increasing numbers of lung transplantations were performed. From about 45 worldwide in 1987, increasing gradually to over 1400 yearly in the mid-nineties, to over 4000 reported yearly to the International Society for Heart and Lung Transplantation (ISHLT) Registry in recent years. A total of over 60000 transplantations had been reported to the registry up until 2016. Over time, the proportion of double lung to single lung transplantations has increased steadily. Numbers of double lung transplantations are increasing and contributing to the upward trend over time in total yearly numbers of performed transplantations, while numbers of single lung transplantations are remaining fairly stable over the years. The continuous upward trend in total yearly numbers of transplantations has plateaued over the last few years.¹³

The most frequent indication for lung transplantation is chronic obstructive pulmonary disease (COPD), with or without α -1-anti-trypsin deficiency, contributing to 36% of all cases, followed by the broad category of interstitial lung disease or pulmonary fibrosis, contributing to 30% of the cases. The third most common indication, in 18% of the cases, is bronchiectasis, most often related to cystic fibrosis. A range of other less common disorders make up the remainder, including pulmonary arterial hypertension, sarcoidosis, lymphangioliomyomatosis, obliterative bronchiolitis, connective tissue disease and cancer.¹³

Over the years, outcome has improved, due to a multitude of factors; better surgical technique, improvements in anaesthesia and intensive care, refined immunosuppressive therapy, more adequate donor and recipient selection. However, outcome in modern days are far from ideal, with chronic rejection now being the main obstacle to better long-term survival.^{14,15} Although short-term and 1-year survival has steadily increased, median survival times have plateaued, with the 5-year survival being similar between 1999-2008 (55%), compared to 2009-2015 (57%). Median unconditional survival time between 1999-2008 was 6.1 years after transplantation.¹³ In certain groups however, long-term median survival over 10 years has been achieved.¹⁶

Lungs, as opposed to other solid organs, have to function more or less directly after transplantation, despite the trauma inflicted during cold ischemia and transportation. The extent of ischemia-reperfusion injury during this process may lead to short-term failure expressed as primary graft dysfunction (PGD) with the development of tissue oedema, pulmonary infiltrates, and reduced oxygenation capacity.¹⁷⁻²⁰ Improvements in lung preservation techniques have come a long way in minimizing these effects.²¹

Shortage of suitable donor organs still remains a major limitation to extending the possibility of lung transplantation to even more patients. Patients are still waiting in vain for new organs, there is a substantial waiting-list mortality, and there are many more patients with co-morbidities that would benefit from lung transplantation but that, due to shortage of donor lungs, currently are not even considered for it. Lung transplantation compared to other solid organs has a relatively low organ procurement rate with only as little as 15-20% of organs being harvested in multi-organ brain dead donors, in the USA and United Kingdom.²²⁻²⁴ Numbers are however higher in certain centres; in Gothenburg data from the time period of 2008-2017 indicates that 36% of multi-organ donors donate lungs. Kotecha et al published a study in 2017 achieving an overall donor use of 41% using extended criteria donor lungs.²⁵ Despite this, there is still a demand-supply mismatch, and lungs from multi-organ-donors are used for transplantation much less often compared to other solid organs such as the liver and the kidneys.

Ideal lung transplant donor acceptability criteria according to the ISHLT were published in 2003.²⁶ The donor should be ≤ 55 years of age; ABO compatible; have a clear chest radiograph; $\text{PaO}_2/\text{FiO}_2$ mmHg > 300 at a FiO_2 of 1.0; a tobacco history of less than 20 pack-years; no chest trauma; no evidence of aspiration or sepsis; no prior cardiopulmonary surgery; a sputum Gram stain without organisms; and absence of

purulent secretions at bronchoscopy. These rather strict criteria together with the lungs' inherent susceptibility to injury, may in many instances lead to a too conservative approach and low utilization rate. Extended criteria accepting organs not fulfilling all above mentioned criteria have been implemented and shown to not result in inferior clinical outcome, thereby increasing numbers of potential donors.^{25,27-29} However, accepting more marginal organs increases risk and may lead to a more complicated post-operative course.^{30,31}

Donation after brain death is currently the largest pool of donors. Several factors during the death-process and the subsequent management of the brain-dead donor can contribute to lung injury. Trauma, aspiration, ventilator-associated pneumonia and sepsis, ventilator-induced lung injury through volume- and barotrauma, atelectasis, oxygen toxicity and volume overload are all prevalent causes of injury.

Inherent to the brain-death process is a cytokine storm, inducing a systemic inflammatory response and lung injury similar to that of acute respiratory distress syndrome (ARDS).³² A catecholamine surge during the brain death process, in an attempt by the body to maintain cerebral perfusion, induces severe hypertension with concomitant increased left-sided heart pressures, leading to neurogenic pulmonary oedema.³³

To expand the possible donor pool further, there has been resurgence of interest in donation after cardiac death (DCD).³⁴ In 1995 D'Alessandro et al reported the first successful DCD donor lung transplantations as part of a program with controlled (withdrawal of life support in the ICU) DCD.³⁵ Since then several centres worldwide have introduced DCD programs, primarily in the setting of controlled donation after withdrawal from life support, with encouraging results, as a way of increasing donor numbers.^{34,36} In Sweden DBD has been the only option up until now, however a DCD-programme is under way of being implemented.

Ex vivo lung perfusion

Stig Steen and the early days

During the twentieth century, in conjunction with the development of cardiopulmonary bypass and growing interest in transplantation, perfusion and preservation of organs, perfusion of thoracic organs was studied. Early attempts of perfusing lungs resulted in deteriorating function and oedema formation.³⁷ This era has been thoroughly described by Sanchez et al.³⁸ Normothermic ex vivo lung perfusion (EVLP) in the setting of auto perfusion of the heart and lungs for preservation during distant procurement was studied in a clinical setting by Hardesty et al. in the 80's, but was abandoned due to lower outcomes.³⁹

Stig Steen and colleagues developed EVLP in the latter part of the 1990's in the context of donation from non-heart-beating donors.⁴⁰⁻⁴³ In 2001 they reported, after careful ethical consideration, the successful single lung transplantation of a right lung from a non-

heart-beating donor to a 54-year-old recipient with COPD, after EVLP of the organs.⁴⁴ Crucial to this success was the development of Steen Solution™, a buffered perfusate containing a high albumin concentration with optimal colloid osmotic pressure, which allowed for perfusion at physiologic pressures without the development of pulmonary oedema, later also having been attributed antioxidative and cytoprotective effects.⁴⁵⁻⁴⁷ Steen solution™ have since then been widely used in all three prevailing EVLP protocols, although subsequently, in the OCS Lung protocol Steen solution™ has been substituted. In part due to its high cost, alternative solutions have been investigated.⁴⁸

In 2005 the Lund group performed the first transplantation of an initially rejected lung after seventeen hours of EVLP reconditioning. The donor was brain-dead following a traffic accident and the lung subjected to EVLP was oedematous, with bleeding spots and atelectasis. However transplantation was successful and computed tomography (CT) after three months showed a normal lung.⁴⁹ A following study by the group in 2006 reported the ex vivo evaluation of six donor lungs deemed non-acceptable for transplantation.⁵⁰ In 2009, Ingemansson et al. followed up on these results with a series of 6 patients receiving double lung transplantation after EVLP, from nine marginal donor lung pairs evaluated with EVLP.⁵¹

A second study evaluating EVLP as a method for assessing transplantability was published 2006 by Egan et al.⁵² They evaluated lungs from six brain-dead donors, using a similar setup as the Lund group. In a porcine experimental setup Erasmus et al. used EVLP to evaluate two different clinical non-heart-beating donor protocols, and whether EVLP could be used for 6 hours of ex vivo preservation. They experienced stable gas exchange during this period of EVLP, but pulmonary artery pressures and ventilation pressures were increased.⁵³

In 2008 Cypel et al. in Toronto published a paper on extended EVLP assessment of lung function using a new protocol, the “Toronto protocol”,⁵⁴ differing in several aspects from the one developed earlier by Steen et al., being the starting point for one of the more influential EVLP strategies. It put a stronger emphasis on the reconditioning properties of EVLP, aiming to extend the timeframe of EVLP, and in 2009 they published a study comparing extended cold preservation with prolonged (12 hours) EVLP.⁵⁵

Rational and indications for clinical EVLP

The rationale behind EVLP is to acquire a window of time to evaluate and assess lungs of marginal quality, outside the body of the donor, when it is believed that organ pathology could be reversible, using this time to “recondition” the organs.^{50,56} In this way, it is possible to evaluate, and in many cases subsequently transplant many organs that previously would have been discarded, thereby substantially increasing the availability of transplantable organs.^{57,58}

Neurogenic pulmonary oedema developing in DBD donors is reversible,^{33,59} however measures taken to minimize it in vivo, using diuretics and fluid restriction, may not always

be successful. During EVLP, the lungs are re-warmed to body temperature and perfused with a hyperoncotic solution, believed to promote the clearing of oedema during the EVLP procedure.⁵⁰ However, experiences from Gothenburg somewhat questions this hypothesis, as lungs may both gain or lose weight during EVLP.⁶⁰ In an effort to further enhance the oedema-reducing properties of EVLP, Wallinder et al. published a case-report on haemofiltration during EVLP.⁶¹

Further, during EVLP, it is possible to directly examine the tissues for any signs of trauma, tumour, infection or atelectasis, as well as to perform a collapse-test assessing the compliant properties of the lungs. It is possible to under direct vision re-expand any atelectatic tissue, atelectasis that in vivo often is not being responsive to recruitment manoeuvres. Bronchoscopy can be performed, the bronchial tree inspected for evidence of inflammation, infection and secretions, and bronchial secretions can be cleared and sent for further analysis. The lungs can be weighed and conclusions drawn from weights before and after EVLP.³⁸

Pulmonary vascular resistance can be calculated from pulmonary artery pressures and perfusate flow measurements and followed over time. Ventilatory variables such as airway pressures and calculated lung compliance can be recorded and followed over time. Perfusate samples can be taken for blood-gas analysis and further testing.

Criteria for EVLP differ between institutions. However, gross trauma and established irreversible lung damage is considered a contraindication. In the study in 2012 by Cypel et al., lungs without evidence of irreversible lung damage, with an oxygenation index ($\text{PaO}_2/\text{FiO}_2$) less than <300 mmHg, and radiologic signs of extensive lung oedema were included.⁵⁶ In Gothenburg, lungs with a $\text{PaO}_2/\text{FiO}_2$ of less than <40 kPa with radiologic signs of infiltrates suggestive of pneumonia, lung infarction, or aspiration were considered for EVLP.¹

The EVLP circuit and the clinical protocols

The typical EVLP system include a perfusion circuit with a pump, a gas-exchange module, a heater-cooler unit for heating or cooling the perfusate, a leukocyte filter,⁶²⁻⁶⁴ a reservoir and tubing connecting the parts together, along with cannulas connecting the circuit to the lung (Figure 1). Also included is an endotracheal tube connecting the lungs to a ventilator and a gas supply (both oxygen and air to the ventilator, and a de-oxygenating gas to the gas-exchange module incorporated into the circuit). A temperature probe for recording perfusate temperature is included as well. Left atrial return of perfusate from the lungs can be either collected passively into a reservoir (Lund protocol) or through a closed cannula sutured to the left atrial remnant (Toronto protocol). Pumps can be either roller, centrifugal or piston-operated.

Initially constructed with in-house supplies from the perfusionist's department, four different commercialized devices have now been developed for EVLP, incorporating all necessary technology into one unit, together with disposable materials adapted for each

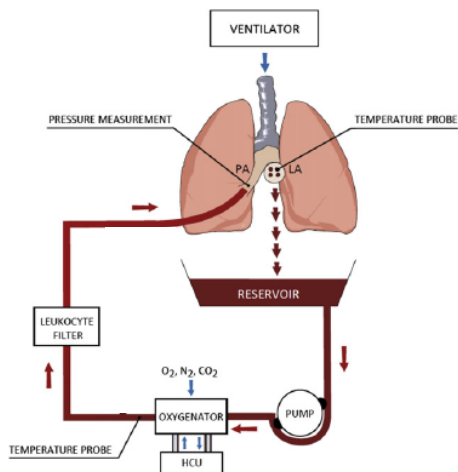


Figure 1.

A schematic drawing of the typical EVLP unit.

Adapted from Wallinder et al¹

PA (pulmonary artery), LA (left atrium), HCU (heater/cooler unit).

system. These devices are the Vivoline LS1 (Vivoline), the XPS (XVIVO Perfusion AB), Lung assist (Organ assist) and Organ Care system Lung (OCS). They all apply a certain protocol of EVLP, according to Lund, Toronto or OCS.

Three predominant protocols for the execution of EVLP have been developed (Table 1, adapted from Andreasson et al⁶⁵ and Maksidi et al⁶⁶). The original, developed in Lund by Stig Steen et al. and the protocol developed by the Toronto group, differ in the use of red blood cells in the perfusate fluid, the flow at which evaluation is performed, the type of pump used, and whether pulmonary venous blood drains passively from the lungs in an open circuit or through a closed circuit with both pulmonary artery (PA) and left atrial (LA) cannulas, and the application of a LA pressure.^{67,68} The Toronto protocol advocates a low flow strategy and acellular perfusate, whereas the Lund protocol employs red blood cells added to the perfusate and evaluates at physiological perfusate flows. All protocols include corticosteroids to suppress immune response and broad-spectrum antibiotics in the perfusate to treat any concomitant infection.^{69,70}

In both the Lund and the Toronto protocols, lungs are initially harvested, cold-flushed and kept in static cold storage, before being subjected to EVLP. After completion of EVLP, awaiting transplantation, lungs are usually cooled and put in cold storage, however this practice has been questioned.⁷¹ OCS differs in that aspect, providing EVLP opportunities immediately after harvesting and pulmonary flush, during transportation, thereby intending to minimise lung ischemia time.⁷² Protocols have over time been adapted and developed locally and differ from centre to centre in some aspects.

The decision to proceed with transplantation after EVLP is based on the performance of the organs in several different aspects: an adequate lung oxygenation capacity with an adequate PaO₂/FiO₂ ratio (>40 kPa/>300 mmHg) during the evaluation phase; stable

hemodynamic and respiratory variables (pulmonary vascular resistance (PVR), peak airway pressures, and lung compliance), the absence of any macroscopic signs of pneumonic infiltrates, lung infarctions or other gross pathology, and a normal collapse test indicating normal elastic and compliant properties of the lungs.

Table 1 - Three different protocols for EVLP

| | Lund | Toronto | OCS |
|--------------------------|--------------------------|----------------|----------------------------|
| Perfusion | | | |
| Target flow | 100% of CO, 70 ml/kg/min | 40% of CO | 2-2.5 l/min |
| PA pressure (mmHg) | ≤20 | ≤15 | ≤20 |
| LA pressure | open LA | 3-5 | open LA |
| Type of pump | Roller | Centrifugal | Pulsatile |
| Perfusate | Steen Solution with RBC | Steen Solution | OCS lung solution with RBC |
| Ventilation | | | |
| Tidal volume (ml/kg) | 6-8 | 7 | 6 |
| Frequency | 10-15 | 7 | 10 |
| PEEP (cm H2O) | 5 | 5 | 5 |
| Fraction inspired oxygen | 0.5 | 0.21 | 0.21 |
| Temperature °C | | | |
| Start of ventilation | 32 | 32 | 32 |
| Start of perfusion | 15 | 25 | 32 |
| Start of evaluation | 37 | 37 | 37 |

PA = pulmonary artery; LA = left atrium; CO = cardiac output; RBC = red blood cells; PEEP = positive end-expiratory pressure

Clinical results - the Lund protocol

The Lund protocol was primarily designed as an assessment system, evaluating lungs under physiologic conditions after rewarming, given a sufficient time for reconditioning. The lungs are recruited and ventilated, bronchoscopy performed, the inflammatory burden decreased by a leukocyte filter added to the circuit, time given for antibiotics and glucocorticoids to have their effect, and time given for the hyperoncotic perfusate to reduce oedema. Time on EVLP is not spent longer than needed if the organs are deemed acceptable for transplantation, however time can be extended if there still is potential for further improvement.^{1,49,73} The initial clinical series published by Ingemansson et al. has been described above.⁵¹ The same group followed up with a review of their clinical experience in 2011.⁴⁵

Wallinder et al. published early results from a clinical EVLP program in Gothenburg, based on the Lund protocol, in 2012.¹ Soon after that, an EVLP program based on the same protocol was initiated in Copenhagen.⁷⁴ Wallinder et al. have in several publications presented the outcome compared to a contemporary control group of conventional lung transplantations.^{60,75} Long-term outcome in patients receiving lungs after EVLP, have in these studies been shown to be comparable to conventional lungs.

Valenza et al. in Milano, published in 2012 a small study consisting of two patients on ECMO receiving marginal lungs after EVLP reconditioning, compared to a small contemporary control of conventional transplantations, with similar results between groups despite the critical state of the recipient population.⁷⁶ They returned in 2014 with another study reporting transplantation after EVLP in seven cases compared to 28 controls with no significant differences in outcome and survival.⁷⁷

Fildes et al. in Manchester compared early outcome up to one year after transplantation in a group of 9 patients transplanted with lungs after EVLP compared to a temporary control group without finding any significant differences in early clinical outcome between the two groups.⁷⁸

Fisher et al. published results from the Develop UK study in 2016.⁷⁹ In a non-randomized study of transplantation after EVLP versus standard-criteria lungs, only 18 out of 53 donor lungs were subsequently transplanted, and results showed a non-significant lower one-year survival in the EVLP, as well as, higher rate of early graft injury and postoperative ECMO support, at increased cost.

Clinical results - the Toronto protocol

The Toronto protocol, developed by Keshavjee and colleagues, has, since its introduction, gained widespread use all over the world.^{54,56,57,80-82} In addition to the evaluative possibilities of EVLP, focus is also on prolonging the perfusion with the possibility of treating and better reconditioning the organs.⁸³ This approach is reflected in their early publication on repair of human donor lungs by IL-10 gene therapy.⁸⁴ Just recently, the Toronto group, almost a decade later, published a study on the same subject, in a large animal model, demonstrating that ex vivo treatment with AdhIL-10 is safe and improves post-transplant lung function after EVLP.⁸⁵

With a view to improving the Lund protocol, they apply acellular perfusate with the intention to avoid damaging haemolysis, a lower target flow to reduce any oedema formation and a closed circuit with the maintenance of a positive LA pressure through a specifically adapted LA cannula.⁸⁶

Being the first prospective clinical trial in EVLP, the HELP trial, published by Cypel et al. in 2011 was ground-breaking.⁵⁶ Twenty out of 23 sets of lungs considered high-risk were transplanted and outcome compared to a group of 166 patients receiving conventional lungs. No differences were seen with regards to primary graft dysfunction (PGD), days on ventilator, time in ICU or hospital- and 30-day mortality. In 2012 the same group reported their experience of 50 consecutive transplantations after 58 EVLP evaluations (86% conversion rate), with lungs from both DCD and DBD donors, compared to a contemporary control group.⁵⁷ Short-term outcome were similar in both groups, and one-year survival was 87% in the EVLP group compared to 86% in the control group.

Aigner et al. in Vienna published a study in 2012 on 9 double-lung transplantations after EVLP assessment, compared to a control group of 119 patients, reporting similar short-term results between groups.⁸⁷ The same year, Zych et al. in Harefield, after having published a case report on a successful transplantation after EVLP in 2011,⁸⁸ reported transplantation of 6 pairs of donor lungs out of 13 EVLP evaluated, with a 100% 3-month survival rate.⁸⁹

A retrospective study of independently collected data from Toronto, Paris and Vienna, encompassing 125 EVLP evaluations was presented at the ISHLT meeting in 2013.⁹⁰ Both DCD and DBD donors were included in the cohort. Lungs were transplanted in 103 cases, mounting up to a conversion rate of 82.5%. Good short- and intermediate-term outcome was reported, with a one-year survival rate of 88%.

The Paris group published their cohort in 2014, consisting of 31 double lung transplantations after 32 EVLP evaluations, compared to a contemporary control group. They experienced significantly longer times in ICU and in hospital stay in the EVLP group, but one-year survival was excellent in both groups, 93% and 91% respectively.⁹¹

Turin reported no difference in incidence or severity of PGD in eight cases of transplantation after EVLP compared to a control group of 28 patients.⁹²

The result of the NOVEL trial,⁹³ a prospective, non-randomised trial in six centres in the USA was presented in 2014.⁹⁴ 76 EVLP:s were performed and 42 were transplanted (55% utilization rate), and compared to a contemporary control group. Early outcome after lung transplantation and one-year survival were not significantly different between patients that received EVLP compared to standard criteria lungs.

Maryland reported short-term outcome data in 11 transplanted grafts out of 17 evaluated with EVLP (65% conversion rate). There were no severe PGD at 72 hours in their material.

In 2017, in a retrospective study of data from the Toronto Lung Transplant Program database, Yeung et al. compared outcome in patients receiving lungs exposed to EVLP for more than 12 hours (mean 14.6 hours) compared to a control group with shorter EVLP-times than 12 hours (mean 6.7 hours).⁹⁵ Longer EVLP did not significantly affect short-term outcome. This is in line with the Toronto approach to EVLP, viewing EVLP not just as a means of evaluation, but also as a treatment opportunity, and a method of increasing flexibility around the transplantation process.

In 2017 Slama et al. published a study comparing two groups of donor lungs, that both met standard inclusion criteria for transplantation.⁹⁶ One of the groups underwent EVLP prior to transplantation, and the other group was transplanted in a standard fashion. Functional results and perioperative outcome in the EVLP group were comparable to those achieved with standard donor lung preservation techniques. It was concluded that EVLP is an option to safely extend total preservation time.

Under way is the large multicentre “Normothermic EVLP As An Assessment Of Extended/Marginal Donor Lungs study”.⁹⁷

Although short- and intermediate-term outcome has been widely published, long-term data is scarcer. In 2015 the Toronto group published a retrospective study of all transplants performed between 2008 and 2012 and reported up to five-year survival-rates that were similar between groups, as were freedom from chronic lung allograft dysfunction (CLAD), rejection episodes, 6-minute walk distance and FEV₁.⁹⁸

Clinical results - Hannover/Madrid – OCS

In 2012, Warnecke et al. presented a new application of EVLP, evaluating the normothermic preservation and transportation of standard criteria lungs on a portable EVLP system, the Organ Care system™ Lung (Transmedics, Andover, USA).⁷² Twelve pairs of standard lungs were preserved under normothermic conditions in this study, as opposed to traditional cold preservation. Short-term outcome was non-inferior compared to control.

The OCS protocol, in line with the Lund protocol, applies a cellular perfusate and open LA, but in agreement with the Toronto protocol, limit flows. As opposed to the other two protocols, Steen Solution has been replaced by a solution made by the system manufacturer.

The principal initial focus of OCS was the transportation of standard criteria lungs using normothermic EVLP, setting it apart from the Lund and Toronto protocols that were primarily designed as protocols for evaluating and reconditioning marginal donor lungs, in-house. The INSPIRE-trial was a prospective randomized multicentre study comparing outcomes of standard criteria lungs preserved and transported by either normothermic EVLP in the OCS system or standard cold preservation.^{99,100} The use of the OCS Lung in the reconditioning of marginal lungs was studied in the EXPAND trial, results of which are to be presented at the 2018 ISHLT meeting. In 2016, Zerouh et al. reported a study comparing short- and medium-term outcome in a group of 14 organs preserved with the OCS Lung, compared to a control group of 308 patients transplanted after cold preservation. Patients in the OCS group had a significantly better postoperative FEV₁ at 3 and 6 months, and similar outcome in terms of cumulative survival and freedom from bronchiolitis obliterans syndrome (BOS).¹⁰¹ Schmack et al. published a recent review in 2016 on the OCS Lung concept.¹⁰² The focus of this thesis is, however, on the Lund and Toronto protocols and their application.

EVLP in special cases

Brown et al. reported a case of EVLP on donor lungs with pulmonary embolism, in which lung oxygenation capacity increased and PVR decreased during EVLP, suggesting that they could have been acceptable for lung transplantation.¹⁰³ A second case report on the same subject, using urokinase during EVLP, and subsequently successfully transplanting a pair of lungs was published in 2014.¹⁰⁴ Daine et al. published a case on successful

transplantations after EVLP in a series of five DBD donors after asphyxia by hanging.¹⁰⁵ Patil et al. used EVLP to evaluate lungs that inadvertently during the harvesting procedure were flushed with very high PA pressure.¹⁰⁶ Costa et al. evaluated EVLP of lungs from 12 donors with a history of cardiac surgery with successful results and outcome.¹⁰⁷ De Wolf et al. used EVLP to gain time while waiting for a negative result in cross matching in hyperimmunized patients, then moving forward with transplantation in three patients, with all patients alive after three years.¹⁰⁸

Establishing the baseline in human EVLP and predicting outcome

A series of descriptive publications with the primary aim of establishing baseline parameters during EVLP have been presented. There has over time also been a growing interest in investigating factors and markers during EVLP, predicting successful outcome after transplantation.

In 2011, Sadaria et al. established a cytokine expression profile in human lungs undergoing normothermic EVLP.¹⁰⁹ The same year, Koike et al. reported on lactate metabolism, during acellular normothermic EVLP, concluding that a gradual increase due to reduced clearance was to be expected.¹¹⁰ In 2013, George et al. presented physiologic and biochemical profiles of clinically rejected lungs on EVLP.¹¹¹

In 2015 Machuca et al. in Toronto, using perfusate samples from 50 EVLP procedures in human lungs collected after 1 and 4 hours of EVLP, investigated the expression of cytokines, chemokines and growth factors. They showed that perfusate biomarkers could potentially be used for more precise donor lung selection improving outcome after transplantation.¹¹² In a contemporary study they evaluated the endothelin-1 pathway as potential predictors for lung function.¹¹³

There is a great interest in finding biochemical markers or combinations of markers during EVLP that could predict the transplantability of the organs. None has so far reached clinical use. Research is active in this field, and a range of preliminary smaller studies have been published during 2017.¹¹⁴⁻¹¹⁸

EVLP as an experimental platform in animal studies

Although having been extensively applied in clinical practice for the last decade, animal studies was the first step, binding theory and practice together, needed for the development of the protocols later to be tested in human research. EVLP is still a novel technique in constant development, and a steady stream of experimental animal studies into different aspects of EVLP have emerged in parallel with clinical progress over the last decade. In this section a brief description of the findings of the majority of these experimental studies is given.

Because of their similarity to human lungs, albeit not identical, porcine lungs have been the most extensively used.^{119,120} There are, however, also experimental studies performed on lungs from other animals, such as rat, mice and dog.¹²¹⁻¹²⁵

Emamina et al. investigated in a small study in 2011 the use of an adenosine A_{2A} agonist during EVLP, suggesting a positive effect on oedema and ischemia-reperfusion injury, confirming these findings in a study in mice in 2015.^{123,126} In 2016, on the same theme, they published a second study in mice on the attenuation of ischemia-reperfusion injury by an adenosine A_{2B} antagonist.¹²⁷ Several recent studies have reported positive results after adding agents to the perfusate, for example by inhibiting NF- κ B¹²⁸ or by adding trimerazidine to attenuate ischaemia/reperfusion injury during EVLP.¹²⁹ None of these experimental approaches has yet been implemented into clinical practice.

Meers et al. published in 2011 the work on an aspiration model in swine and demonstrated the feasibility of assessing the organs by EVLP.^{130,131} They then used this model to compare steroids to macrolides to improve gas-exchange in caustic-injured lungs.¹³² In 2014 Khalife-Hocquemiller et al., in an aspiration-model with gastric acid, found that surfactant attenuated lung injury during EVLP.¹³³ Last year in a similar study in pigs, Nakajima et al. reported better outcome after lung lavage and surfactant replacement during EVLP after gastric-acid induced lung injury.¹³⁴

Valenza et al. investigated glucose consumption during EVLP, and found that increased glucose consumption correlated with worse lung function.¹³⁵ Following up on these results, in 2012, they showed that salbutamol infusion during EVLP was associated with lower pulmonary pressures and better lung mechanics.¹³⁶ These findings were confirmed in a canine study by Kondo et al. in 2015, showing that inhalation of salbutamol attenuates lung injury.¹²² A third study in 2017, in a canine model, by Hijiya et al. supported earlier findings.¹³⁷

The Toronto group performed prolonged EVLP during 12 hours in porcine lungs subjected to 24 hours of cold ischemia, whereby they organs were transplanted and evaluated in vivo.¹³⁸ The organs developed oedema during EVLP and decreased compliance, and subsequently performed poor in vivo. The significant finding in this study was that ex vivo PO_2 is a poor indicator of lung performance due to the linearization of the relationship between oxygen content and PO_2 in acellular perfusate. The same group published an additional study, the same year, comparing ex vivo with in vivo intratracheal adenoviral vector gene delivery.¹³⁹

The poor reliability of PO_2 alone in assessing lung function during EVLP is well known, especially using acellular perfusate, as shown in the above-mentioned study. Conditions are different using cellular perfusate, however, it has been shown that it is important to relate the PaO_2/FiO_2 ratio to the inspired fraction of oxygen.¹⁴⁰ PaO_2 is related to shunt fraction and depending on the extent of shunt the relationship between PaO_2 and different FiO_2 varies.¹⁴¹ Pablo et al. in Maryland have adapted their EVLP protocol to put stronger emphasis on compliance dynamics in decision-making during EVLP.¹⁴² Okamoto et al. studied correlations between the PaO_2/FiO_2 ratio and airway and vascular parameters during cellular EVLP in both porcine and human lungs and found that airway parameters were complementary quantitative indicators of lung function in cellular EVLP.¹⁴³

Thrombolytic therapy during EVLP in a rat model was studied in 2013 by Motoyama et al., and again in 2014.^{144,145} It has, as described above, been applied in clinical cases as well.

Maignan et al. have studied measurement of exhaled carbon monoxide (CO) during EVLP, as a marker for ischemia reperfusion injury, showing a positive correlation between exhaled concentration of CO and the degree of ischaemia/reperfusion injury.^{146,147}

Pierre et al. evaluated in a porcine model the effects of extending EVLP-times, reporting increasing weight with increased time on EVLP. Results from this study could not in an experimental setting show benefit from additional time on EVLP, contradicting later clinical reports of successful outcome after extended EVLP in human lungs.¹⁴⁸

Noda et al. have published several experimental studies in rat, beginning with the establishment of a rat EVLP model in 2014.¹²⁴ The same year they demonstrated that lung grafts on EVLP exhibited prominent pro-inflammatory changes and compromised metabolic profiles. It was possible to attenuate this by inhaling the lungs with hydrogen.¹⁴⁹ In a study in 2015, they compared a novel technique of dual EVLP, also including the bronchial artery circulation to the EVLP circuit, achieving superior outcome in this group.¹⁵⁰ Recently they published a study on optimal O₂ levels in the perfusate during EVLP. Deoxygenated perfusate exhibited significantly more inflammation with compromised cellular metabolic activity and compromised post-transplant outcomes.¹⁵¹

In 2015, Harada et al. reported on a small study assessing the addition of a neutrophil elastase inhibitor to the perfusate, indicating that the inflammatory response may be attenuated and lung reperfusion injury be decreased.¹⁵² Lin et al. reported in 2017 positive results of adding alpha-1-antitrypsin to the perfusate.¹⁵³

In a study in rat, thermography was used to detect regional malperfusion during EVLP, in lungs with induced thrombi. Thermographical evaluation may, in contrast to prevailing modalities, detect regional damage in donor lungs.¹⁵⁴ In 2017 Sage et al. reported on using real-time CT to assess organs during EVLP.¹⁵⁵

Martens et al. in a study in 2016 could not, in a porcine model, improve graft function using inhalation of argon or xenon gases during EVLP.¹⁵⁶ In a succeeding study by the same group, exposing lungs to maximal xenon exposure, they again did not find support for this approach.¹⁵⁷ Several studies have found support for protective effects of inhaling different agents during EVLP, such as sevoflurane.¹⁵⁸

Renewed interest in cytokine filtration to reduce accumulation during prolonged EVLP led Iskender et al. to publish a porcine study showing that cytokine removal decreased the development of pulmonary oedema and electrolyte imbalance. These findings contrast with the negative findings by Kakishita et al. back in 2010.¹⁵⁹

Lately there has also been an interest into ventilation strategies applied during EVLP. In 2017, Mehaffey et al. demonstrated superior results with airway pressure release ventilation during EVLP.¹⁶⁰ The same year, Aboelnazar et al. presented a negative pressure ventilation model, where they could demonstrate decreased inflammation and less oedema formation during EVLP.¹⁶¹

In 2017 Himmat et al. showed that a decrease in hypoxic pulmonary vasoconstriction during EVLP correlated with increased inflammation during extended EVLP.¹⁶²

Although most experimental studies have been performed according to either the Lund or Toronto protocol, some studies adopting OCS have emerged. Among them a study in prolonged EVLP using OCS Lung and comparing cellular and acellular perfusates.¹⁶³

EVLP in the context of DCD

There is growing support of the use of EVLP after controlled DCD donation.^{57,164-166} In 2015 Machuca et al. published a study evaluating the impact of EVLP on outcome after transplantation of donation after DCD lungs.¹⁶⁷ Between 2007 and 2013, out of a total of 673 lung transplantations performed, 55 were DCD transplantations (excluding bridged cases from analysis in this study). Twenty-eight (51%) of the DCD cases underwent EVLP. Outcome after transplantation with organs from DBD compared to DCD donors was similar, up to five years after transplantation. Organs from DBD donors that were transplanted after EVLP presented shorter hospital stay and a trend towards shorter time on ventilator, but without any difference in survival.

EVLP has been extensively used as an experimental platform evaluating different procedures and approaches to performing transplantation of lungs after DCD.¹⁶⁸⁻¹⁷⁴ EVLP after DCD is however outside the scope of this thesis and will not be covered further.

AIMS

General aims

The general aims of this thesis were to increase the understanding of ex vivo lung perfusion (EVLP) the way it is contemporarily performed according to two different strategies, to experimentally investigate refinements and additions to the procedure and to compare two established protocols.

Additional aims were to review the clinical outcome of patients that had been transplanted with lungs that had undergone EVLP in two Scandinavian centres, and to investigate whether it could be established which parameters during EVLP matter the most for clinical outcome.

Study aims

- I. To evaluate the effect of haemofiltration during EVLP on lung function, perfusate oncotic pressure, and lung weight as a measure of tissue oedema content, in a porcine model.
- II. To compare two clinically used strategies for EVLP with respect to lung function, metabolism, inflammatory response, oxidative stress, and cell viability, in a porcine model.
- III. To review the combined clinical short- and medium-term outcome of patients undergoing lung transplantation after EVLP in Gothenburg and Copenhagen and compare it to a contemporary control group of patients transplanted with conventional (non-EVLP) lungs.
- IV. To assess the correlations between several physiological variables during EVLP, with short-term outcome variables in 47 double lung recipients of EVLP-evaluated lungs, to identify which factors are the most important in predicting outcome after transplantation.

METHODS

The animal experiments were performed at the Laboratory for Experimental Biomedicine, at Gothenburg University. Paper II is in collaboration with representatives of the Laboratory for Microbiology, Parasitology and Hygiene, Antwerp University, Antwerp, Belgium and the Department of Thoracic Surgery, Medical University of Vienna, Austria. Paper III and VI are in collaboration with Rigshospitalet in Copenhagen and the University of Copenhagen.

Ethical considerations

Papers I and II – experimental animal studies

The experimental animal studies were performed following approval of The Animal Ethical Committee of the University of Gothenburg. The animals received care in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986), 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 from the National Institutes of Health, USA. At the end of the experiments animals were euthanized by exsanguination.

Papers III and IV – clinical studies

The Human Ethics Committees of the University of Gothenburg and the University of Copenhagen approved the clinical studies. All patients were informed, both orally and in writing, and consented in writing, when listed for transplantation, to the possibility of receiving organs that had undergone EVLP. Participants were allowed to withdraw from the study at any time. The organs were matched according to standard criteria.

Animal studies (papers I and II)

Animals

Swedish domestic pigs were supplied by a local breeder. All animals were delivered to the laboratory one week in advance of performing the study. A veterinarian supervised care and handling of the animals, before and during experiments.

Twenty-two animals were included in Paper I, of which sixteen were subjected to induction of pulmonary oedema *in vivo*, and six controls were not. Two animals in the oedema induction group were excluded due to them developing severe right heart failure

and the inability to maintain study protocol targets. The remaining fourteen animals were randomized, in two equally sized groups, to EVLP either with or without haemofiltration.

Twenty animals were included in Paper II, randomized to two equally sized study groups.

Measurements during the experiments were when appropriate indexed to body surface area.¹⁷⁵

Anaesthesia and preparation

The animals were fasted overnight, with free access to water. They received premedication while in stables, with an intramuscular injection of a combination of 0.06 mg/kg body weight (BW) dexmedetomidin (Dexdomitor, Orion Pharma AB, Sollentuna, Sweden) and 5 mg/kg BW, respectively, of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil, Virbac, Carros, France).

The animals were then transferred to a preparation room and placed in the prone position. They were given an intramuscular injection of buprenorphine 0.03 mg/kg BW (Vetergesic vet, Orion Pharma Animal Health, Sollentuna, Sweden) and an intravenous catheter was placed in the ear. Tracheal intubation was performed under spontaneous breathing. The pigs were subsequently transferred to the operating room and placed in the supine position on the operating table.

Volume-controlled ventilation was initiated with a tidal volume of 10 ml/kg, a positive end-expiratory pressure (PEEP) of 0 mmHg (Paper I) or 5 mmHg (Paper II), and a FiO_2 of 0.5, adjusting the respiratory frequency to maintain end-tidal CO_2 (et- CO_2) in the normal range of 5-5.5 kPa, using a Servo Ventilator (Servo Ventilator 900C, Siemens-Elema AB, Solna, Sweden). Anaesthesia was maintained throughout the experiment using isoflurane (Isoba Vet, Intervet AB, Sollentuna, Sweden) at a minimal alveolar concentration (MAC) of 1.3.

Lung harvesting

A cannula was inserted in the pulmonary artery and secured in place with a purse string suture, and the left atrial appendage was incised widely to allow for free drainage of the organ preservation fluid. The lungs were then perfused antegradely at a low perfusion pressure (<20 mmHg) with 2 litres of cold Perfadex (Vitrolife AB, Gothenburg, Sweden) with added isotonic trometamol, 0.35 ml (Addex-THAM 3.3 mmol/ml, Fresenius Kabi AB, Uppsala, Sweden), calcium gluconate, 1.4 ml (9 mg/ml) and nitroglycerin, 3 ml (5 mg/ml, BMM Pharma AB, Stockholm, Sweden), to every litre of Perfadex. The lungs were harvested in a standard fashion and subsequently perfused retrogradely with an additional 2 litres of cold Perfadex with the above-mentioned additives. The lungs were weighed again and put in a bag of cold Perfadex. They were then stored at 8 °C for two hours.

Following 2 hours of cold ischemia the lungs were again weighed and again retrogradely perfused with an additional 1 l of Perfadex with the above-mentioned additives.

Induction of pulmonary oedema (paper I)

An arterial catheter was placed in the right femoral artery, using ultrasound-guided Seldinger technique. The same method was used to place an 8.5 Fr catheter introducer in the right external jugular vein, through which a Swan-Ganz catheter (Swan-Ganz CCOMbo, 7.5 F Edward Life Sciences, Irvine, California) was introduced and forwarded to the pulmonary artery under observation of the pulse pressure wave form. A temperature probe was placed orally.

A median sternotomy was performed, and the pericardium was opened. A balloon-tipped catheter was placed in the left atrium (LA) through a small incision in the left atrial appendage and secured in place with a purse string suture. (Figure 2).

An infusion of Ringers-acetate was initiated at a rate of 20 ml/kg BW/hour. The LA balloon was inflated gradually, while maintaining a pulmonary artery systolic pressure (PAs) of at least 50 mmHg, until a pulmonary capillary wedge pressure (PCWP) of 25-30 mmHg was reached. The PCWP was then maintained in this range for the duration of two hours, adjusting the volume of the left atrial balloon as needed. Arterial blood gases (ABL 725 Radiometer, Copenhagen, Denmark), cardiac output (CO), heart rate, mean arterial pressure (MAP), PAs, mean pulmonary artery pressure (PAm), diastolic

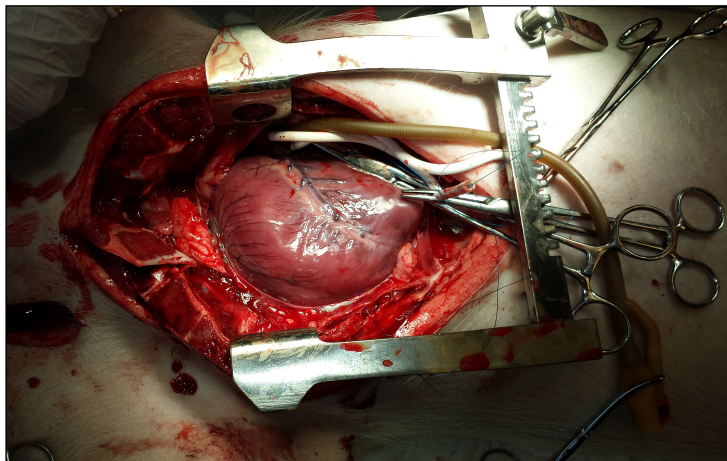


Figure 2.

Image depicting an animal after sternotomy and placement of the left atrial balloon-tipped catheter, through the left atrial appendage. The catheter is yellow in colour and can be seen at the top of the image, rounding the left lateral border of the heart. The heart is markedly rotated to the left and the left atrial appendage cannot be seen in this picture.

pulmonary artery pressure (PA_d) and PCWP, as well as, et-CO₂, tidal volume and inspiratory plateau pressure, were measured and recorded (BeneView T8, Mindray Medical Int Ltd, Shenzhen, China) after sternotomy, before and after the induction of lung oedema. CO was measured using the cardiac output module of the BeneView T8, by bolus injections of cold saline. At each recording, CO was averaged over three measurements. The animals were then exsanguinated to a cell saver (Autolog, Medtronic Inc, Minneapolis, USA) and the blood processed for use during the EVLP phase of the experiment.

The lungs were harvested as described above and randomized, in blocks of four, to haemoconcentration by haemofiltration (HF) of the EVLP perfusate (n=7, HF group), or no haemofiltration (n=7, control group), during EVLP reconditioning.

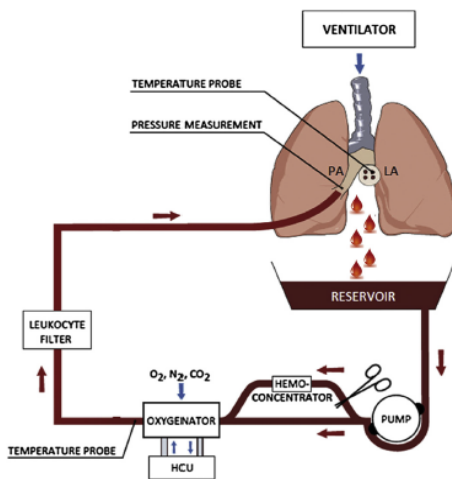


Figure 3.

A schematic drawing of the typical EVLP unit with the option of haemofiltration of the perfusate. PA (pulmonary artery), LA (left atrium), HCU (heater/cooler unit).

Haemofiltration during EVLP (paper I)

A schematic overview of the EVLP circuit is found in Figure 3. Settings during different phases of the experiment are found in Table 2.

Perfusate: EVLP was performed with the Vivoline LS1 EVLP system (Vivoline Medical AB, Lund, Sweden) (Figure 4). The system was primed with 2 l of Steen Solution (Vitrolife AB, Gothenburg, Sweden) and mixed with salvaged and washed red cells from the pig to a haematocrit of 10-15%. Imipenem, 500 mg (Meronem, AstraZeneca, Södertälje, Sweden), 10000 IU Heparin (LEO Pharmaceutical Products Ballerup, Copenhagen, Denmark) and methylprednisolone 1 g (Pfizer AB, Sollentuna, Sweden) were also added to the perfusate. The pH of the solution, measured after 10 minutes of circulation in the system, was corrected for every unit below zero in base excess with 1 ml of isotonic

trometamol plus an additional 5 ml empirically needed to achieve a pH of 7.35-7.45. After an additional 10 minutes of perfusion, the pH was measured again, and additional trometamol was added if needed.

Perfusion: The lungs were prepared for EVLP with a custom-fitted cannula in the main pulmonary artery and a tube in the trachea; both secured in place with cable ties and connected to the outflow line from the EVLP unit and the ventilator (Figure 5). The remnant of the left atrium was opened widely to prevent pulmonary vein outflow obstruction. A temperature probe was sutured inside the left atrium. PA-pressure was measured continuously with a catheter in the main pulmonary artery and was calibrated to zero to the bottom of the lung evaluation box. Pressure, as well as flow, could be regulated, and the perfusate temperature set to a specified degree, with a maximum temperature difference between lung in- and outflow perfusate not allowed to exceed 8°C during warming.

The Vivoline LS1 is automated and has one phase for lung reconditioning and one for evaluation of lung function. During reconditioning, the oxygenator is supplied with a gas mixture of nitrogen 74%, oxygen 21% and carbon dioxide 5%. During the evaluation phase (see below), the oxygen supply is disconnected, and the oxygenator is used to deoxygenate the perfusate in the EVLP system with a gas mixture of 93% nitrogen and 7% carbon dioxide. Flow during the reconditioning phase was set to a maximum of 40 ml/kg/min and a minimum of 20 ml/kg/min.



Figure 4.

The Vivoline LS1 during ex vivo lung perfusion, together with the ventilator at the left of the picture.

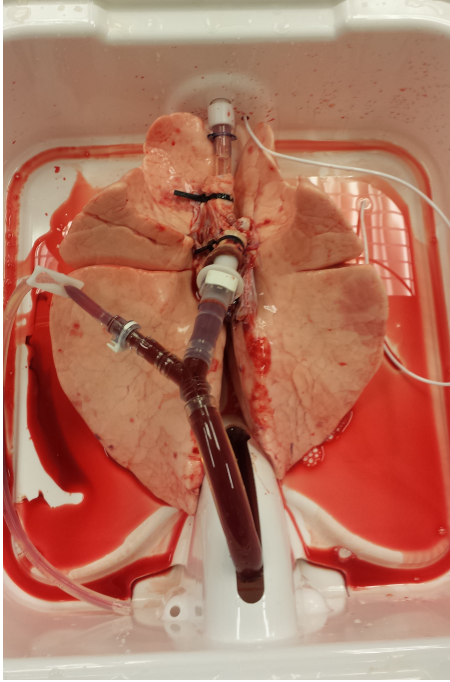


Figure 5.

Porcine lungs during EVLP. The cannula to the PA is seen in the centre of the image. The tube to the trachea is at the top of the image.

Rewarming: When attached to the outflow cannula and the ventilator, having the temperature probe attached, perfusion was initiated in reconditioning mode and the temperature set to 15°C with the pulmonary artery pressure set to a maximum of 15 mmHg and the maximum flow to 2-4 ml/kg BW/min. The shunt from the inflow cannula was closed after assuring that the perfusate was completely de-aired. Once the shunt was closed the temperature was set for 36°C, and the maximum flow increased to 4-8 ml/kg BW/min. This was maintained until 32°C was reached, when the maximum flow was increased to 6-12 ml/kg BW/min and pressure controlled ventilation was initiated, with a peak inspiratory pressure of 12 mmHg, PEEP 5 mmHg, a frequency of 7/min and a FiO_2 of 0.5. At 34 °C, the maximum flow was increased to 10-20 ml/kg BW/min.

First lung evaluation: When 36 °C was reached the lungs were recruited at a pulmonary perfusate flow of 0, with a PEEP of 15 mmHg. The ventilation was then set to a tidal volume of 6 ml/kg BW, a respiratory frequency of 10/min, a PEEP of 5 cm H₂O and a FiO_2 of 0.5. The system was set in evaluation mode, and the lungs were perfused with either a maximum PA pressure of 25 mmHg or a maximum flow of 6 l/min (the maximum flow of the pump). After 5 and 10 minutes, blood gases were drawn from the left atrium (outflow) and after the gas exchanger (inflow). Haemodynamic and respiratory variables were recorded. In addition, oncotic pressure of the perfusate was measured at five minutes after the start of the evaluation phase (Osmomat 050 Colloid Osmometer®, Gonotec GmbH, Berlin, Germany).

Reconditioning with/without haemofiltration: After the first evaluation, the system was again set in reconditioning mode with a perfusate flow of 20-40 ml/kg/min and a maximal PA pressure of 25 mmHg. Ventilation was continued with a tidal volume of 6 ml/kg body weight at a PEEP of 5 cm H₂O and a respiratory frequency adjusted to obtain a minute ventilation 1.5 times the pulmonary artery flow. A haemofilter (Hemocor HPH 400; Medivators, Minneapolis, Minnesota) was attached to the circuit in seven EVLP circuits after randomisation (HF-group), with the aim of a total ultrafiltrate volume of 500 ml. The reconditioning phase continued for a total of 180 minutes. The lungs were recruited at a PEEP of 15 cm H₂O every 60 minutes. Blood gases were drawn from left atrium and from the gas exchanger every 60 minutes and 1 ml of isotonic trometamol was given for every unit below zero in base excess.

Table 2 – Settings during the different phases of the EVLP procedure.

| Stage of experiment | Rewarming | | | 1st evaluation | Re-conditioning | 2nd evaluation |
|--|-----------|---------|---------|------------------------|-------------------------------------|------------------------|
| | -32°C | 32-34°C | 34-36°C | | | |
| EVLP device phase¹ | Recond | Recond | Recond | Evaluation | Recond | Evaluation |
| PA pressure | 15 | 15 | 15 | 25 | 25 | 25 |
| PA flow range² (ml/kg BW/min) | 4-8 | 6-12 | 10-20 | max 6 l/min | 20-40 | max 6 l/min |
| Ventilation (FiO ₂ 0.5, PEEP 5) | | | | | | |
| Mode | - | PC | VC | VC | VC | VC |
| PIP / Vt | - | 12 mmHg | 12 mmHg | 6 ml/kg BW | 6 ml/kg BW | 6 ml/kg BW |
| f | - | 7 /min | 7 /min | 10 /min | Adjusted to obtain MV 1.5 x PA flow | 10 /min |
| Lung recruitment (PEEP 15 mmHg) | | | | At start of evaluation | Every 60 minutes | At start of evaluation |

¹ The EVLP device has one phase for reconditioning (oxygenator gas flow: nitrogen 74%, oxygen 21% and carbon dioxide 5%) and one for evaluation (oxygenator gas flow: nitrogen 93% and 7% carbon dioxide).

² Flow was restricted at different levels according to the stage of experiment. During evaluation flow limits were set to the maximum of the EVLP device, 6 l/min.

FiO₂ = fraction of inspired oxygen, PEEP = positive end-expiratory pressure, Vt = tidal volume, MV = respiratory minute volume, PA = pulmonary artery, PC = pressure-controlled ventilation, VC = volume-controlled ventilation, BW = body weight, Recond = reconditioning, f = frequency.

Second lung evaluation: After 180 minutes, the system was again set in evaluation mode with the same flow and respiratory settings as during the first evaluation. Perfusate samples were taken, and recordings of haemodynamic and ventilatory variables were performed as described above. A bolus dose of nitroglycerine (15 mg) was administered to the perfusate at the end of the experimental procedure, followed by new measurements of haemodynamic and respiratory variables five minutes later. The lungs were then disconnected, the main pulmonary artery cannula and the tracheal tube were removed, and the lungs were weighed.

EVLP protocol comparison (paper II)

Two experiments, one in each arm of the study, were performed each day in parallel. The animals each day were randomized to either the ACA group (n=10) or the COA group (n=10).

The animals were exsanguinated to a cell saver as described previously (COA group) or to waste (ACA group). The lungs were then harvested as described above, with the following addition after retrograde perfusion: Tissue samples were taken from the inferior parts of the basal lobe of either the left or right lung in a randomized fashion. From this tissue, samples were secured for quantitative PCR (qPCR) and quantification of reactive

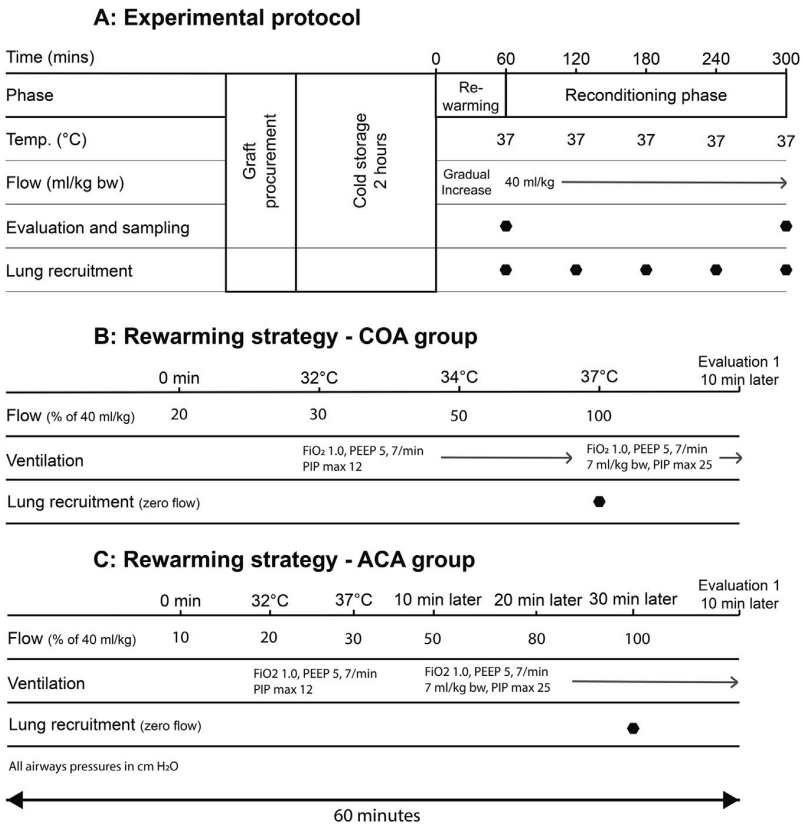


Figure 6.

(A) Experimental protocol for EVLP, (B) Rewarming protocol for the COA group,

(C) Rewarming protocol for the ACA group.

bw = bodyweight; FiO₂ = fraction of inspired oxygen; PEEP, positive end-expiratory pressure; PIP, peak inspiratory pressure.

oxygen species (ROS) in tissue. The lungs were again weighed and stored in cold Perfadex at 8°C for two hours. After additional retrograde perfusion with 1 litre of Perfadex, preparations for EVLP were done according to the respective protocols, described below. An overview of the experimental protocol and rewarming strategies for the respective protocol is found in Figure 6.

At the end of EVLP the lungs were again weighed, and tissue samples were secured for wet-dry weight ratio, qPCR, quantification of ROS and histopathological analysis.

A collapse-test was performed in conjunction with the disconnection of the tracheal tube. Lung collapse was classified, subjectively, as normal or impaired, by one of the authors (TN) in all cases.

The accessory lobe was selectively cannulated and perfused with a solution containing trypan blue, followed by a formaldehyde fixative. A tissue sample was then taken from the accessory lobe and sent for histological analysis.

Ex vivo lung perfusion – COA group

Priming: EVLP was performed with the Vivoline LS1 system primed with 1.5 l of Steen solution and added salvaged red blood cells to a haematocrit of 10-15%, methylprednisolone, and heparin as described above for paper I.

Perfusion: The lungs were prepared and connected to the EVLP device. After de-airing of the circuit, the lungs were rewarmed to 37°C. Pulmonary flow was allowed to increase gradually as temperature increased to a target of 40 ml/kg BW. Ventilation was initiated at 32°C. When 36°C was reached a lung recruitment manoeuvre was performed and FiO₂ set to 1.0. At 60 minutes after initiation of EVLP the first evaluation was performed, with evaluation performed in evaluation mode. A reconditioning phase of 240 minutes then followed. The lungs were recruited every 60 minutes during temporary arrest of perfusion¹⁷⁶ and arterial blood gases (ABG) were drawn after a period of stabilization from the recruitment manoeuvre. Base excess was corrected with 1 ml of trometamol for every unit below zero. After 240 minutes of reconditioning, the lungs were recruited and FiO₂ set to 1.0. Five minutes later a second evaluation was performed. During both evaluation phases, the lungs were perfused at a constant flow of 40 ml/kg BW/min.

Ex vivo lung perfusion – ACA group

Priming: EVLP was performed with a custom-built system using disposables supplied by XVIVO (XVIVO AB, Gothenburg, Sweden) and a Cardiohelp System (Maquet, Getinge Group, Sweden) used for perfusion (Figure 7). The system was primed with 1.5 l of Steen solution, methylprednisolone, and heparin.

Perfusion: The lungs were prepared and connected to the EVLP device, ensuring adequate de-airing of the perfusate. The left atrium was cannulated, and LAP was continuously maintained at 5 mmHg during EVLP. The lungs were then rewarmed to 37°C, with a

pulmonary flow (Q_p) allowed to increase gradually, as temperature increased, to a target of 40 ml/kg BW/min. Ventilation was initiated at 32°C. When 37°C was reached, a lung recruitment manoeuvre was performed and FiO_2 set to 1.0. Sixty minutes after initiation of EVLP, the first evaluation was performed. 400 ml of the perfusate was then exchanged for fresh Steen solution followed by a reconditioning phase of 240 minutes. 200 ml of perfusate was exchanged for new Steen solution at 60, 120 and 180 minutes. The lungs were recruited every 60 minutes during temporary arrest of perfusion and perfusate samples for blood gas analysis were drawn. At 240 minutes of reconditioning, the lungs were recruited and FiO_2 set to 1.0. Five minutes later the second evaluation followed. During both evaluation phases, the lungs were perfused at a constant flow of 40 ml/kg BW/min.

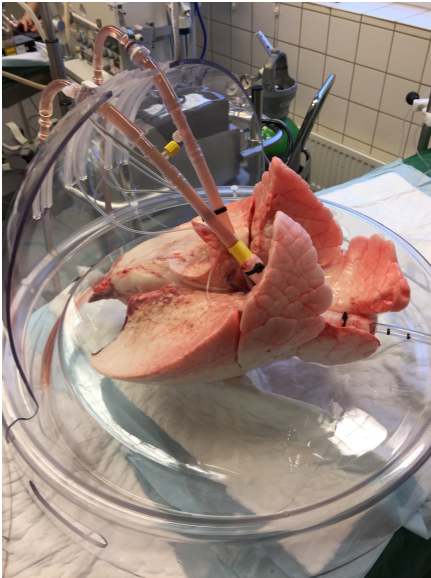


Figure 7.

Porcine lungs during EVLP in the ACA group. The cannula to the PA and the cannula for the return of pulmonary venous blood are seen in the centre of the image. The tube to the trachea is at the right of the image.

Quantification of reactive oxygen species in tissue (paper II)

In collaboration with the Laboratory for Microbiology, Parasitology and Hygiene, Antwerp University, Antwerp, Belgium, electron spin resonance (ESR) was used for the detection of free radical formation generated during EVLP in lung tissue. Vitamin C was used as an endogenous spin probe with a high sensitivity and specificity for intracellular ROS. Snap frozen samples of pig lung were briefly thawed and homogenized (Qiagen TissueRuptor, Qiagen, The Netherlands). Homogenates were placed on ice for direct measurements using ESR spectroscopy. 60 μ L of homogenate was loaded into a quartz glass capillary (Duran Rincaps, Hirschmann, Germany) to acquire the ESR spectrum using a table top MiniScope MS 200 spectrometer (Magnettech, Germany). Results were recorded using Analysis 2.0 (Magnettech, Germany) software and expressed as ESR peak

amplitude arbitrary units (A.U.) as a measure of free radical concentration in the observed sample.

Tissue gene expression (paper II)

Tissue from lungs was harvested at two time points, before and after 4 hours of EVLP. Tissue samples were homogenized, and RNA was extracted using a Maxwell® 16 LEV simplyRNA Tissue kit (Promega, USA) on a Maxwell® 16 MDx Instrument (Promega, USA), according to manufacturer's protocol, by the author.

The Genomics Core Facility, at Gothenburg University, performed the remaining procedures: The RNA concentration and purity were measured using Nanodrop. The RNA quality was evaluated with Tapestation 2200 (Agilent Technologies, USA). cDNA synthesis was performed with a SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Sweden) on a 2720 Thermal Cycler (Applied Biosystems, USA)

Nanodrop II (GC Biotech, The Netherlands) was used to setup the qPCR plates. To run the qPCR, the QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies, Sweden) instrument was used. TaqMan® Gene Expression Master Mix (Life Technologies, Sweden) and TaqMan® Gene Expression Assays (Life Technologies, Sweden), were used for detection of for IL-6, IL-8, IL-10, IL-1beta, TNF-alfa, IFN-gamma, HIF-1alfa, and GAPDH. Everything was performed according to the manufacturer's protocol and reaction volume was 5 µl.

Histopathology (paper II)

Tissue samples were taken for macroscopic examination, from the base of one of the lower lobes, and fixed in formaldehyde. They were embedded in paraffin, sectioned and stained with hematoxylin and eosin. A pathologist blinded to sample identity evaluated the sections with regards to vascular thrombosis, haemorrhage, necrosis, interstitial oedema, intra-alveolar oedema, intra-alveolar fibrin deposition, arteriolar thickening, cell infiltration, peribronchial oedema and cell infiltrate. The severity of these changes was graded as described by Inci et al¹⁷⁷ and scored from 0 (absent) to 4 (severe).

Trypan blue staining to assess cell viability (paper II)

Tissue from the accessory lobe perfused with trypan blue and formaldehyde solution was fixed in formaldehyde solution. Samples were embedded in paraffin, sectioned and stained with eosin. Examination was performed as reported by D'Armini et al.¹⁷⁸ Viable cell nuclei colours pink, non-viable as blue. The ratio of viable to non-viable nuclei was reported.

Clinical Studies (Papers III and IV)

Patients from Copenhagen and Gothenburg were prospectively and consecutively

included between January 2011 and December 2015, and followed until the end of December 2016. DBD donors primarily rejected for transplantation were considered for EVLP. Lungs that, during EVLP, achieved acceptable lung function were transplanted.

Surgery was performed as to local preference and routine, either via bilateral sequential thoracotomy or sternotomy, either with or without extracorporeal circulation. Outcome in recipients transplanted after EVLP or without prior EVLP were compared (Paper III), with regards to short-term outcome expressed as PaO₂/FiO₂ at arrival in ICU, time on ventilator, time in ICU, time to discharge and survival. Medium-term outcome expressed as lung function at one year and survival free from re-transplantation was also studied.

Ventilator time was defined as time to extubation in hours. Time in ICU was defined as the number of days from ICU arrival to general ward discharge. If the recipient was re-intubated during the index procedure hospitalization, ventilator time was defined as the total ventilator treatment time.

Correlations between factors during EVLP and short-term outcome were studied in lungs that were transplanted (Paper IV). Univariate and multivariate linear regression was performed on the seven prognostic variables and the three outcome variables.

Clinical EVLP

EVLP at our two centres was performed based on a modified version of the procedure described by Stig Steen et al.,⁴⁹ and has been extensively described by Wallinder et al.^{1,60,74,75} Equivalent protocols were applied in both institutions, using the Vivoline LS1 device, perfused with Steen solution mixed with red blood cells to a haematocrit of 10-15%, 10000 U of heparin and 100 mg of meropenem. Following lung recruitment manoeuvres and bronchoscopy, evaluation was performed at 36 degrees C, at full perfusate flow and pressure.

Acceptable lung function was defined as: a) PaO₂/FiO₂ ratio >40 kPa (Gothenburg) PaO₂/FiO₂ >50 kPa at FiO₂ 1.0 or Pao₂ >13 kPa at FiO₂ 0.21 (Copenhagen). b) pulmonary vascular resistance (PVR) and pulmonary compliance deemed normal and not deteriorating during EVLP; c) macroscopic appearance and manual inspection without major pathology.

Physiological dead space fraction, static lung compliance and PVR were continuously monitored. A collapse test was performed to evaluate the compliant properties of the lungs and was graded as normal or impaired.

Accepted lungs were surface cooled in the EVLP system awaiting transplantation. Following transplantation patients received care according to local standard practice and protocol.

Statistics

A p-value of <0.05 was considered statistically significant. Inspection of normal Q-Q Plots and the Shapiro-Wilks test were used for testing continuous variables for normal distribution. Statistical analysis was performed using IBM SPSS Statistics for Macintosh, Version 22 and 23 (IBM Corp., Armonk, NY).

Experimental animal studies (papers I and II)

Continuous data were presented as mean and standard deviation. Changes within groups were assessed by Wilcoxon's signed-rank test. The relative changes of the variables between groups were compared using the Mann-Whitney U test. Correlations between variables were evaluated using the Pearson correlation coefficient.

Short- and medium-term outcome after EVLP (paper III)

Continuous data were presented as mean and standard deviation when normally distributed, otherwise as median and range. Categorical data were presented as frequency and/or percentage. Differences between groups were evaluated with the Student's T-test in normally distributed data, otherwise with the Mann-Whitney U-test. Kaplan-Meier curves were used for survival analysis and a log-rank test for comparison of proportional survival between the groups.

Correlation of physiological variables during EVLP with short-term outcome post transplantation (paper IV)

Continuous data were presented as mean and standard deviation and as median and range. Correlations between variables were evaluated using the Spearman's rank correlation coefficient. Univariate and multivariate linear regression was performed on the seven prognostic variables and the three outcome variables.

RESULTS

Animal studies (papers I and II)

Induction of pulmonary oedema (paper I)

Two animals were excluded due to the inability to maintain study targets during induction of lung oedema.

The HF and noHF groups did not significantly differ in body weight or body surface area.

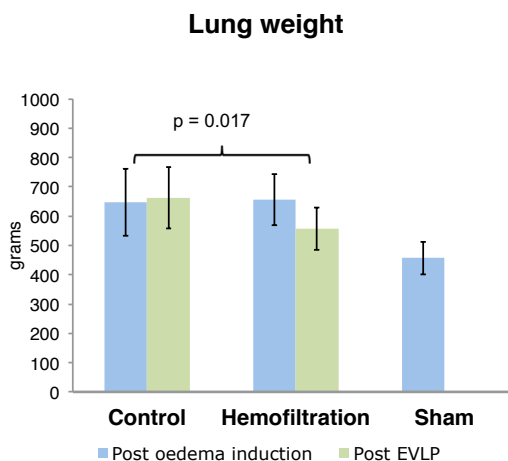
Induction of pulmonary oedema caused a decrease in oxygen saturation, arterial PO_2 , PO_2/FiO_2 ratio and compliance, an increase in arterial PCO_2 and PVRI. The model was successful in inducing a pulmonary oedema. Lung weight after oedema induction was 43% higher in the study group compared to the sham group of six animals (Figure 8).

No significant differences were found between the noHF and the HF groups at the end of oedema induction with respect to oxygen saturation, arterial PO_2 , arterial PCO_2 , left atrial PO_2/FiO_2 ratio, compliance, dead space ratio or PVRI. The same applies to lung weight, lung weight/kg body weight, and lung weight/body surface area.

At first evaluation, compared with in vivo values after pulmonary oedema induction, the left atrial PO_2/FiO_2 ratio increased considerably during EVLP whereas dead space ratio did not change significantly (Figure 9).

Figure 8

Lung weights after oedema induction in the two study groups, and the sham group.



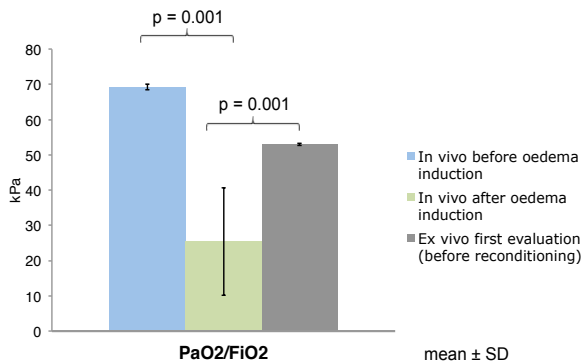


Figure 9.
PaO₂/FiO₂ at different stages of the experiment in paper I.

Haemofiltration during EVLP (paper I)

Haemofiltration during the reconditioning phase significantly increased the oncotic pressure of the perfusate by 43% and the haematocrit by 26%, whereas in the noHF group, there was only a minor increase in oncotic pressure (11%) and the haematocrit was unchanged. Haemofiltration caused a significant decrease of 15% in lung weight; lung weights were unchanged in the noHF group.

Lung compliance decreased significantly in both groups during reconditioning, but significantly more so in the HF group. Changes in Δ PO₂/FiO₂ ratio, left atrial PO₂/FiO₂ ratio, shunt fraction, arterial oxygen saturation and dead space during EVLP did not differ between groups. Both groups had a 55% decrease in pulmonary flow index, and a 2.3-to 2.7-fold increase in PVRI, with no significant difference between groups.

All lungs developed consolidation of the inferior lobes and an impaired collapse test.

A close correlation was found, of both left atrial PO₂/FiO₂ and Δ PO₂/FiO₂ with intrapulmonary shunt fraction ($r=0.71$ and $r=0.78$, respectively).

Nitroglycerine decreased PVRI in the nonHF and HF groups, by 45% and 35%, respectively, and increased pulmonary flow by 88% and 82%, respectively. Nitroglycerine impaired lung oxygenation capacity, as demonstrated by a decrease in the PaO₂/FiO₂ ratio in both groups, as well as a decrease in Δ PO₂/FiO₂ in both groups. With nitroglycerine, intrapulmonary shunt fraction increased by more than half in both groups.

EVLP protocol comparison (paper II)

There were no differences in BW between the two study groups. In three lungs of the ACA group, severe oedema developed during EVLP. The perfusate volume reached such low levels in these three cases that it was not possible to continue EVLP reconditioning as planned. The second evaluation in these cases was therefore not possible to conduct,

and statistics are based on the seven cases in the ACA group in which the protocol could be finalized. EVLP of the lungs in the COA group was performed as planned in all experiments (n=10). Mean lung weights increased significantly in both groups during EVLP. There was a trend for a more pronounced increase in the ACA (44%) compared to the COA group (23%, $p=0.065$).

The transpulmonary oxygen gradient decreased significantly in the ACA group but not in the COA group (Fig. 10). The dead space fraction increased in the ACA but not in the COA group. Compliance decreased significantly in both groups, by 44% in the ACA group, and by 25% in the COA group with a trend for a more pronounced decrease in the ACA group ($p=0.083$). PVRI increased significantly in both groups. This increase in PVRI did not differ between groups. The oncotic pressure increased in both groups during EVLP. This increase was significantly more pronounced in the COA group (20%) compared to the ACA group (9%, $p = 0.001$). All lungs in both groups developed consolidation of the inferior lobes and exhibited an impaired collapse test.

Quantification of reactive oxygen species in lung tissue (paper II)

We could not observe any significant difference between groups in changes in peak-to-peak amplitude measurements of pulmonary ascorbyl radical signal in lung tissue, from the first to the second evaluation ($p=0.436$). Electron spin resonance peak amplitudes (arbitrary units) were $1,070 \pm 234$ before and 965 ± 247 after EVLP in the ACA group ($p=0.475$) compared with 917 ± 123 before and 966 ± 257 after EVLP in the COA group ($p=0.878$).

Tissue gene expression (paper II)

The expression of hypoxia-inducible factor (HIF)-1 α decreased significantly in both groups during EVLP with no difference between groups. The levels of the mRNAs for the proinflammatory cytokines IL-6 and IL-8 decreased to similar extent in both groups, while TNF- α decreased in the ACA group and increased in the COA group ($p=0.003$). The level of the mRNA for the anti-inflammatory cytokine IL-10 decreased in the ACA group but not in the COA group ($p=0.005$).

Histopathology (paper II)

Histologic examination showed mild pathology in a few cases. Mild interstitial infiltrates were noted in two samples in each group. Mild arteriolar thickening was present in two samples respectively in each group. There was no apparent difference in lung histopathology between groups and no relevant statistical analysis could be performed.

Trypan blue staining to assess cell viability (paper II)

There was no statistically significant difference between the groups with regards to the viable/non-viable nuclei ratio.

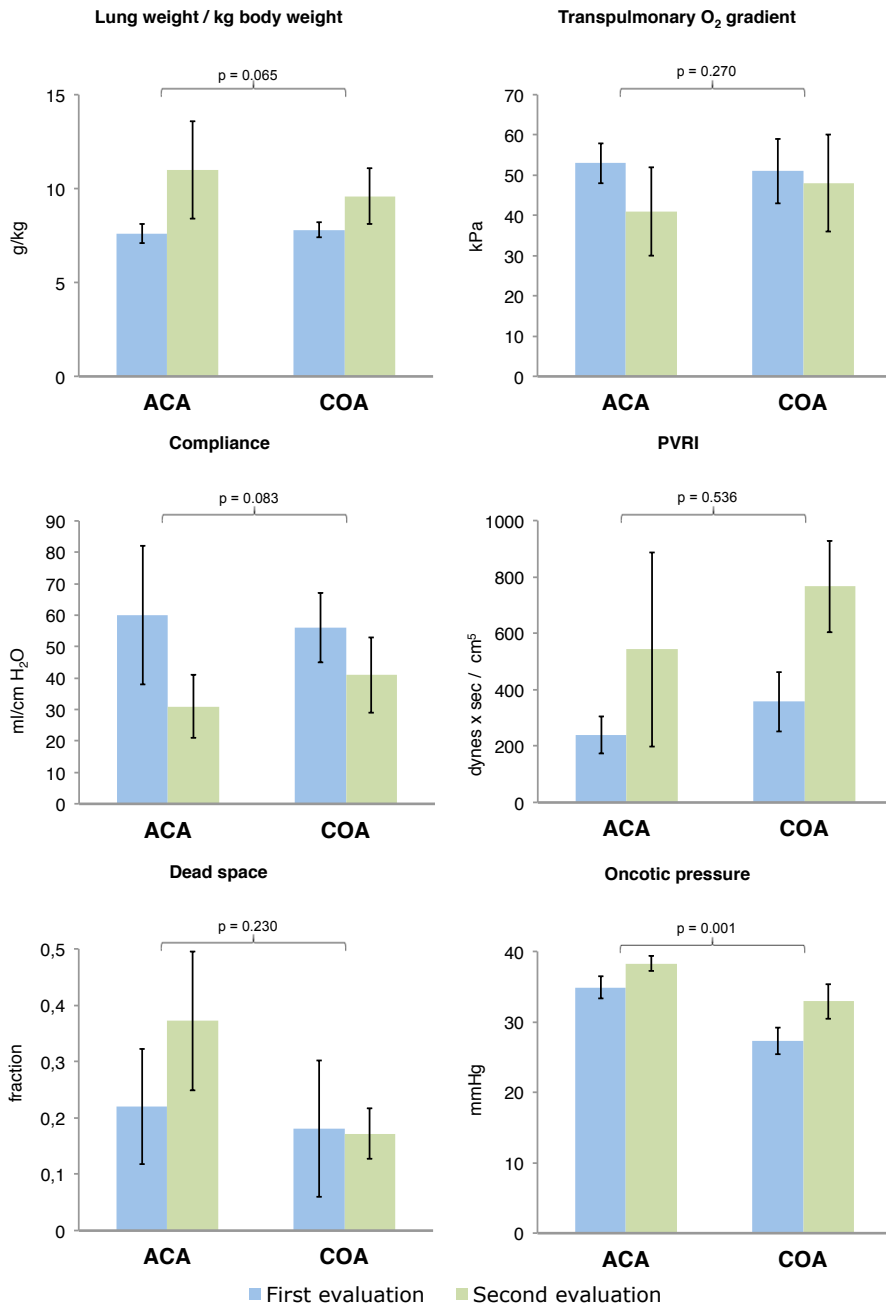


Figure 10.

Mean values of lung weight/kg body weight, transpulmonary oxygen gradient, compliance, pulmonary vascular resistance index, dead space fraction and perfusate oncotic pressure before and after four hours of EVLP reconditioning. P-values indicate difference in change between groups. $p < 0.05$ is considered significant.

Clinical studies (papers III and IV)

Short- and medium-term outcome after EVLP compared to conventional lungs (paper III)

During the four-year study period, from January 2011 to December 2015, with Gothenburg initiating its clinical EVLP program in January 2011 and Copenhagen in May 2012, lungs from 1013 donors were offered to our two centres, and 54 patients underwent lung transplantation with initially rejected lungs but subsequently used after EVLP. All other contemporary lung transplantation (n=271) procedures with organs accepted according to standard selection criteria not requiring EVLP, were included as a control group. Re-transplantations during the study period were excluded. Recipient characteristics between groups were similar, including age of recipients and diagnosis underlying the indication for transplantation. A higher percentage of recipients in the EVLP group were on preoperative ventilatory support, while in the control group a higher percentage were on preoperative ECMO (Table 3).

Table 3 - Recipient characteristics

| Recipient variable | EVLP | Conventional |
|--------------------------------|-----------|--------------|
| Age (years) | 52 ± 12 | 51 ± 13 |
| Diagnosis (%) | | |
| IPF | 24 | 25 |
| PAH | 2 | 6 |
| COPD | 33 | 28 |
| Alfa-1-antitrypsin deficiency | 6 | 13 |
| CF | 20 | 12 |
| Other | 15 | 16 |
| Preoperative ventilator | 5 (9.3 %) | 12 (4.4 %) |
| Preoperative ECMO | 1 (1.9 %) | 16 (5.9 %) |

IPF = interstitial pulmonary fibrosis, PAH = pulmonary artery hypertension, COPD = chronic obstructive pulmonary disease, CF = cystic fibrosis, ECMO = extracorporeal membrane oxygenation.

In our combined cohort, 61 donor lungs underwent EVLP. Forty-seven double lungs were deemed transplantable. In one of these cases the pair of lungs were split and transplanted in two different recipients. In one case bilateral bilobar transplantation was performed. In five cases, one of the lungs was used for single lung transplantation, and the other discarded after EVLP. This results in a conversion rate of 87% (53/61) of all EVLP cases. 246 double lung transplantations and 37 single lung transplantations were included in the control group. The use of intraoperative ECC/ECMO was similar in the two groups.

One patient in the EVLP group and four in the conventional group died within the first 48 hours after transplantation. In none of these cases was death attributable to insufficient lung function. PaO₂/FiO₂ at arrival in ICU was significantly lower in the EVLP group, as were time to extubation and median ICU-stay. Time to discharge to home or rehabilitation did not differ between groups (Table 4).

One-year survival, free from re-transplantation, was 87% (95% CI 82-92%) in the EVLP group and 83% (95% CI 81-85 %) in the conventional group. Cumulative survival, free from re-transplantation during the entire period did, in our combined cohorts, not differ significantly between the groups (Figure 11).

Lung function tests did not show a significant difference in FEV₁% at one year after transplantation (Table 4).

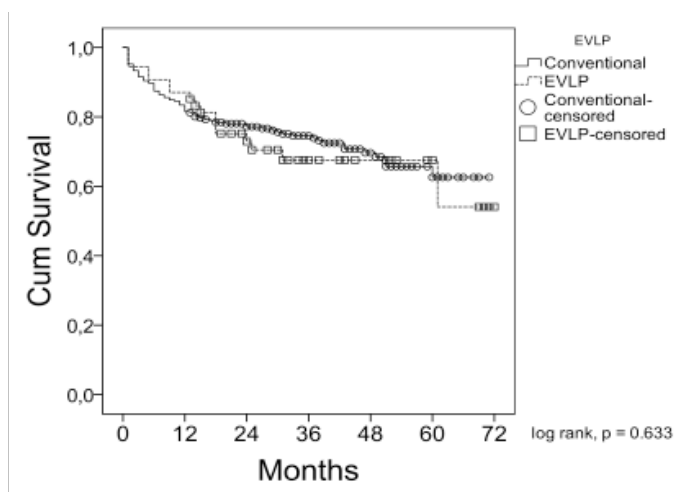
Table 4 - Intra- and postoperative characteristics

| Variable | EVLP | Control | p-value |
|--|-----------------|-----------------|----------------|
| Number of patients | 54 | 271 | |
| Single LTx | 7 (13%) | 37 (14%) | |
| Double LTx | 47 (87%) | 234 (86%) | |
| Intraoperative ECC/ECMO | 29 (54%) | 125 (46%) | |
| PaO₂/FiO₂ at arrival in ICU (kPa) | 30 ± 14 | 36 ± 14 | 0.005 |
| Time to extubation (hours) | | | |
| (median and range) | 18 (2-912) | 7 (0-2280) | 0.002 |
| ICU stay (days) | | | |
| (median and range) | 4 (2-65) | 3 (1-156) | 0.002 |
| Time to discharge (days) | | | |
| (median and range) | 30 (17-112) | 28 (12-268) | 0.35 |
| FEV₁% at 12 months | 75 ± 29 | 81 ± 26 | 0.18 |
| One-year survival | 87% (CI 82-92%) | 83% (CI 81-85%) | |

LTx = lung transplantation, ECC = extra corporeal circulation, ECMO = extra corporeal membrane oxygenation, ICU = intensive care unit. FEV₁% = forced expiratory ventilation in the first second/predicted. PaO₂/FiO₂ = arterial oxygen tension/inspired oxygen fraction.

Correlation of physiological variables during EVLP with short-term outcome after transplantation (paper IV)

During the study period 54 patients received lungs that had undergone EVLP prior to transplantation. In 46 cases both lungs were transplanted, in one case a bi-lobar transplantation was performed and in 7 cases single lung transplantation was performed.



| Number at risk | | | | | | |
|----------------|-----|-----|-----|----|----|----|
| Control | 271 | 226 | 150 | 93 | 42 | 11 |
| EVLP | 54 | 43 | 26 | 13 | 8 | 4 |

Figure 11.

Survival and freedom from re-transplantation after lung transplantation.

In those patients receiving bilateral lung transplantation, correlations between commonly recorded variables and physiologic parameters during EVLP, and clinical outcome, were tested for correlations against PaO₂/FiO₂ at arrival in ICU, recipient time to extubation and recipient time in ICU. No significant correlations were found. (Table 5). There were no significant uni- or multivariate regression models that predicted poor outcome.

Table 5 - Correlations between variables during EVLP and outcome measures

| | PaO ₂ /FiO ₂ at arrival in ICU | | Recipient time to extubation | | Recipient time in ICU | |
|--|--|-------|------------------------------|-------|-----------------------|-------|
| | r _s | p | r _s | p | r _s | p |
| PaO ₂ /FiO ₂ (1.0) (kPa) at EVLP | -0.060 | 0.708 | -0.110 | 0.487 | -0.052 | 0.741 |
| Compliance (ml/cm H ₂ O) | 0.090 | 0.572 | -0.279 | 0.074 | -0.059 | 0.708 |
| Dead space fraction | -0.246 | 0.132 | 0.040 | 0.811 | 0.017 | 0.918 |
| Pulmonary vascular resistance [(dynes x s)/cm ⁵ /m ²) | 0.041 | 0.793 | -0.022 | 0.890 | -0.112 | 0.469 |
| Lung weight change (g) | 0.257 | 0.137 | -0.084 | 0.631 | -0.087 | 0.616 |
| Lung weight at end of EVLP (g) | -0.181 | 0.297 | -0.020 | 0.910 | 0.076 | 0.660 |
| Duration of EVLP (hours) | -0.109 | 0.486 | -0.062 | 0.694 | -0.088 | 0.571 |

PaO₂ = partial pressure of oxygen in arterial blood; FiO₂ = fraction of oxygen in inspired air; EVLP = ex vivo lung perfusion; ICU = intensive care unit

DISCUSSION

Ex vivo lung perfusion has during the last decade firmly been established at a wide range of transplantation centres around the globe as a method of evaluating marginal organs and increasing numbers of transplantable organs. This thesis deals with both experimental and clinical aspects of EVLP.

In the first experimental study (paper I), pulmonary oedema was successfully induced using left atrial outflow obstruction and crystalloid loading. Haemofiltration during EVLP was established as a method of increasing the oedema reducing properties of EVLP, as previous clinical experience had shown that lungs may both gain and lose weight during standard EVLP.⁶⁰ Adding a haemofilter to the circuit and filtrating the perfusate could uphold oncotic pressures, promoting clearance of tissue oedema.

In paper II, a comparison was made between the two prevailing methods for EVLP, the Lund and the Toronto protocol, as they are clinically adapted and performed in Gothenburg and Vienna respectively.^{1,87} The original method, the Lund protocol has been superseded by the Toronto protocol in numbers of performed clinical EVLP:s.⁶⁵ Previous studies have reported on comparisons on specific differences between the protocols, such as acellular to cellular perfusate.⁶⁸ However, in this study, the two protocols were compared head-to-head the way they are clinically performed, in the setting of a standard EVLP evaluation, with a time frame of four hours. Lung edema formation and decreased lung compliance occurred with both EVLP techniques but were more pronounced in the ACA group. Otherwise, there were no differences in lung function, inflammatory response, ischemia/reperfusion injury, or histopathological changes between the EVLP techniques. A novel method in the porcine setting of evaluating cell viability using trypan blue staining was introduced.

Short-term outcome after EVLP has been widely published, although medium- and long-time follow up is scarcer. A thorough account of publicised clinical results has been made in the introductory chapter. Paper III reports on short- and medium-term outcome after EVLP compared to a contemporary control group, in collaboration between Sahlgrenska University Hospital, Gothenburg, Sweden, and Rigshospitalet in Copenhagen, Denmark. Although short-term outcome differs with respect to time on ventilator and time in ICU, the groups showed similar results in lung function at one year as well as survival, free from re-transplantation. The material is one of the larger published to date and the most comprehensive using the Lund protocol.

EVLP evaluation and the final decision to accept organs for transplantation is a synthesis of a wide range of variables and factors. In addition to physiological variables such as PaO₂/FiO₂, compliance and PVR and their dynamics during EVLP, the touch, feel and

look of the organs, as well as the history of the donor are integrated into the decision to proceed with transplantation. Paper IV searched for factors during EVLP that could predict short-term outcome in the patient, however there was no clear correlation between commonly registered variables during EVLP and early clinical outcome.

Ethical considerations

The relevant Regional Ethical Review Boards approved all experimental and clinical studies.

A prerequisite for the ethical acceptability of performing animal studies for scientific purposes is that the expected value of gained information from the experiments outweighs any potential suffering of the animals, and that relevant information cannot be gained in other ways, *in vitro*, or in human studies. Into account must also be taken the fact that the results of animal experimental studies often cannot be directly extrapolated to human clinical conditions, and that there is often a discordance between animal studies and clinical trials.¹⁷⁹ The two animal experimental studies of this thesis could not have been performed on human lungs, due to the lack of suitable human organs, or *in vitro*. The principle of the 3Rs (replace, reduce, refine) in animal scientific experiments was adhered to in the planning and setting up of the experimental protocols.

All animals received care in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986), 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 from the National Institutes of Health.

A veterinarian supervised animal handling and care before and during the experimental procedures. The animals were professionally handled with care and respect by all involved personnel, unnecessary discomfort and suffering minimized, thereby optimizing conditions for reproducible experimental results and minimizing unnecessary loss of animals. All animals were euthanized by exsanguination under general anaesthesia.

In the clinical studies (papers III and IV) all patients were informed and gave oral and written consent, when listed for transplantation, to the possibility of receiving organs that had undergone EVLP. The organs were otherwise matched according to standard criteria. The patients could unconditionally withdraw from the studies at any time.

Experimental studies (paper I and II)

Lung oedema model (paper I)

The model of inducing oedema by means of left atrial outflow obstruction and crystalloid infusion was successful. Compared to a sham group of six animals, where lungs were harvested without prior oedema induction and weighed prior to EVLP, lungs subjected

to oedema induction weighed 43% more, corresponding to a gained oedema volume of about 300 ml. The model was stable and predictable, however care had to be taken to gradually increase the left atrial outflow obstruction and PCWP, else the animals were susceptible to the development of right heart failure, which, once established, proved to be very difficult to manage. Two animals were excluded for this reason.

Reduction of pulmonary oedema by haemofiltration during EVLP (paper I)

Haemofiltration during EVLP, initially described by our group, significantly increased both perfusate oncotic pressure and haematocrit, increasing the forces promoting a fluid shift from perivascular and interstitial space to the vascular lumen. A significant decrease in lung weights after EVLP in the group subjected to haemofiltration indicate that up to a third of the induced oedema could be relieved.

The non-significant increase in lung weight together with the small increase in oncotic pressure in the group without haemofiltration during EVLP indicate a fluid shift from perfusate to tissues, with the development of oedema during EVLP. This contradicts the perception of Steen Solution as having potent oedema-reducing properties. It also contradicts the practice of intermittently exchanging perfusate during EVLP, as practiced in the Toronto protocol, as this will decrease the overall oncotic pressure of the perfusate during the course of EVLP, and therefore increase the tendency of oedema development.

Although the effect of haemofiltration on lung weight and hence oedema content was significant, this did not, in this study, translate into better lung performance expressed as PaO₂/FiO₂. Compliance decreased in both groups during EVLP, but more so in the non-haemofiltration group. All lungs developed basal atelectasis and exhibited a pathological collapse test upon completion of EVLP.

Impact of prolonged EVLP on haemodynamics and compliance

In both experimental papers, across protocols, there was a tendency for PVR to gradually increase and compliance to decrease during EVLP. Uniformly there was development of basal atelectasis and a pathological collapse test, despite hourly lung recruitment manoeuvres during EVLP. Except for in the haemofiltration group of paper I, there was also a tendency for increased lung weights after EVLP, and hence increased pulmonary oedema. Most excessively so in the Toronto arm of paper II, where in three cases, EVLP had to be ended prematurely due to the development of excessive pulmonary oedema and lack of perfusate in the circuit.

Although prolonged EVLP in porcine lungs has been described by Cypel et al, the same group in 2012 reported on the development of oedema during EVLP, decreased compliance and subsequently poor performance in vivo after transplantation, in porcine lungs transplanted after prolonged ischemia and 12 hours of EVLP.^{54,138} Pierre et al described increasing weight of lungs with increased time of EVLP, and could not show any improvement in lung grafts as a result of additional EVLP times.¹⁴⁸

This tendency to develop oedema during EVLP, with deteriorating haemodynamics and compliance over time, is not uniformly seen during EVLP in human lungs where successful prolonged EVLP has been reported repeatedly using the Toronto and OCS protocol.^{163,180,181} The Gothenburg group reported that lungs may both gain and lose weight during clinical EVLP.⁶⁰ Other research groups have in personal correspondence reported the same experiences in experimental setups applying porcine models. This difference in behaviour during EVLP has to be taken into account when planning and interpreting results in porcine lungs. However, porcine models for EVLP is established as one of the best parallels to humans. It is size appropriate with comparable respiratory and hemodynamic settings as in humans, making it possible to use the same technical equipment in experimental studies as in clinical practice. Also, the immune system and biology are similar to humans, and the availability and cost of animals is reasonable.

Oedematous lungs are heavier due to additional fluid content. In the supine position, the degree of lung inflation at any point along the dorso-ventral axis is dependent on the weight of the tissue compressing it from above. The more oedematous the lungs are, the more prone they will be to development of basal atelectasis. In addition, in the context of EVLP there is no negative pleural pressure supporting the lungs. This tendency to develop basal atelectasis might be counteracted by a more active approach in keeping all lung tissue recruited, for example by using a higher level of PEEP, and, if it could be technically feasible during the process of EVLP, turning the lungs over to promote recruitment of alveoli and counteract any atelectatic tendency, in analogy with the clinical practice of ventilating patients in the prone position.

PVR increased gradually over the course of EVLP, and this increase could be counteracted using a vasodilating agent, nitroglycerine. There may be several reasons for this tendency. It could partly be explained by a hypoxic pulmonary vasoconstrictive response to the development of poorly ventilated lung segments in conjunction with atelectasis. It could be hypothesized that the use of red blood cells in the perfusate may increase this response by the inactivation of nitric oxide by haemoglobin,¹⁸² and maybe also due to the release of free haemoglobin in the cell salvage procedure as a result of haemolysis. Free haemoglobin binds nitric oxide, which acts as an endogenous vasodilator, thereby inducing pulmonary vasoconstriction.¹⁸³ However, in the comparison between acellular and cellular perfusates by Roman et al, there was no difference in PVR between groups.⁶⁷

PaO₂/FiO₂ for the evaluation of lung function

The left atrial PaO₂/FiO₂ (PF) ratio has been extensively used as an indicator of lung function in patients with acute lung injury, and others. The PF-ratio is included as one of the parameters in the ISHLT definition of the ideal donor,²⁶ and all EVLP protocols aim to achieve normal ratios during EVLP. In vivo, the ratio is affected by FiO₂, intrapulmonary shunt fraction and mixed venous oxygen content.¹⁴¹ In both experimental studies, the transpulmonary oxygen gradient, ΔPaO₂/FiO₂ was reported. It is the difference in oxygen tension between the venous blood entering the lung and the

oxygenated blood leaving the lungs divided by the inspired oxygen fraction, with the intention of excluding the influence of any differences in mixed venous oxygen content entering the lung. In paper I, a close correlation between calculated shunt fraction and both $\Delta\text{PaO}_2/\text{FiO}_2$ and $\text{PaO}_2/\text{FiO}_2$ were shown, indicating that both variables could be used to assess the degree of ventilation/perfusion mismatch, and hence the degree of lung injury.

The Lund protocol adds red blood cells to the perfusate in contrast to the Toronto protocol, which uses acellular perfusate. Analyses of $\text{PaO}_2/\text{FiO}_2$ in acellular perfusate cannot reliably be used to assess lung function, especially in the presence of a large mismatch in ventilation/perfusion.¹³⁸ Therefore, in Gothenburg, we continue to use cellular perfusate and evaluate at full flow. Roman et al. did not find any significant differences in physiologic, immunologic, or ultrastructural parameters in a porcine experimental study, comparing cellular to acellular perfusate.⁶⁷

Being a rather blunt tool, since $\text{PaO}_2/\text{FiO}_2$ is influenced by a variety of factors, and performing poor as an indicator of lung performance in EVLP using acellular perfusate, focus has shifted to other variables during EVLP, such as compliance.^{140,142,143,184} However, all variables continue to add important information to the overall picture, and decision to go ahead with transplantation after EVLP is ultimately a synthesis of a number of factors.

A novel parameter in the assessment of lung function during EVLP is the PF-difference. It is the difference between PaO_2 at two different FiO_2 , and is dependent on shunt fraction, with decreasing PF difference with increasing shunt and hence poorer lung performance. Niikawa et al. will report on this parameter in an abstract to the 2018 ISHLT meeting as a variable for assessing transplant suitability in EVLP. Further studies will be needed to assess whether it is a useful addition and will find its way into clinical practice.

Comparison of EVLP protocols (paper II)

Although being the first protocol described, the Lund protocol has been challenged by the Toronto protocol, which now is the clinically most applied.¹⁸⁵ Philosophies differ; the Lund protocol's primary aim is evaluation, spending no more time than necessary on EVLP. The Toronto protocol has a different approach appreciating the therapeutic capabilities of EVLP, aiming for extension of EVLP times in order to give a specific treatment time to achieve its effect. Recent years research has been focused on EVLP as a means of extending the timeframe between donation and transplantation.^{96,181} In addition to these different views on the fundamental role of EVLP, the protocols differ with respect to the use of cellular or acellular perfusate, open or closed atrium, the kind of pump used in the circuit and the pressures and flows aimed for during EVLP.^{44,54}

In previous publications these differences have been studied from certain aspects, for example acellular versus cellular perfusate by Roman et al and Becker et al.^{67,68} The Toronto group has expressed belief in the importance of maintaining a positive LA pressure, using a specifically adapted catheter, by monitoring outflow pressures during

the EVLP procedures.⁸⁶ Paper II in this thesis is the first head-to-head comparison of these two protocols in the setting of a normal EVLP-evaluation within a time frame of four hours.

Our research group has substantial experience in performing EVLP according to the Lund protocol, being applied both in clinical practice, and used for experimental research. To ensure that the Toronto arm of paper II was executed correctly, we collaborated with a representative of the Vienna EVLP program in the planning and execution of the study protocol. To study ischemia/reperfusion injury and detect free radicals in tissue, we collaborated with a research group in Antwerp, Belgium, experienced in this field.^{186,187}

Taking into account the development of severe pulmonary oedema in three cases in the Toronto arm, biasing the results of the remaining seven cases, and the trends towards a more pronounced increase in lung weight and decrease in lung compliance, albeit not statistically significant, there was a tendency towards a more pronounced accumulation of oedema in the Toronto group. There was also a fall in oxygenation capacity, and an increase in dead space, in the Toronto group, but not in the Lund group. However, the differences between groups were not significant.

Lung fluid accumulation during the EVLP process, with a fall in compliance and impaired oxygenation capacity could be explained by increased microvascular permeability caused by ischemia/reperfusion injury.^{186,188} Results in paper II are in line with those of paper I, and with clinical experience in Gothenburg,¹ and has been reported by others.^{68,148} The same target pulmonary flow was achieved in both study groups and hence over-perfusion could not be blamed for oedema development. Perfusion with acellular perfusate could theoretically lead to progressive tissue hypoxia in the Toronto group due to the lack of red blood cells. Studies have shown hypoxia-inducible factor 1 α (HIF-1 α) to increase substantially during EVLP.^{149,150} However, during the course of EVLP, expression of HIF-1 α decreased in both groups, contradicting this hypothesis.

As a consequence of ischemia/reperfusion injury, immune cells generate ROS and proinflammatory cytokines, leading to increased vascular permeability and the development of pulmonary oedema.¹⁸⁸ In this study there was no significant difference in tissue ROS content before and after EVLP, and the levels did not differ between EVLP techniques. The expression of the proinflammatory cytokines IL-6 and IL-8 were down-regulated during EVLP, while expression of TNF- α was down-regulated in the Toronto arm, and up-regulated in the Lund arm. Anti-inflammatory cytokine IL-10 expression increased in the Lund arm and decreased in the Toronto arm. Taken together there is no support for the assumption that inflammation-induced vascular permeability could be blamed for the development of oedema in either group.

There were no differences between groups with regards to histopathological findings. Although an established method in experimental research, the histopathological findings were sparse in view of significant pulmonary oedema development and atelectasis, and no significant differences in lung ultrastructure could be seen.

The introduction of trypan blue staining of the accessory lobe via selective perfusion proved to be feasible, yielding interpretable histopathological material. To the authors knowledge, this is the first report of the application of this method in a porcine experimental setting, it has previously only been reported in rodents.¹⁷⁸ There was no significant difference in cell viability between groups.

As in paper I, perfusate oncotic pressure increased during EVLP, due to extravasation of fluid and the development of oedema. The practice in the Toronto arm of intermittently replacing part of the perfusate with fresh Steen Solution may actually reinforce the tendency of extravasation, not allowing the oncotic pressure of the perfusate to increase to the same extent. This could be one of the reasons for the development of excessive pulmonary oedema in the Toronto arm of the study.

In the study from 2013, the Toronto group showed that a closed atrium, maintaining 5 mmHg of outflow pressure, during EVLP led to less oedema.⁸⁶ The assumption is that a continuous positive outflow pressure will lead to a beneficial distribution of perfusion away from dependent regions of the lungs. However, increasing outflow pressure with a constant inflow pressure, transmural gradients will increase in the ex vivo setting, leading to fluid extravasation. Although established as a part of the Toronto protocol, there is still a need for further research before any firm conclusions can be drawn about its superiority over the open atrium technique.

Limitations

In addition to the above-mentioned limitations regarding the extrapolation of results in animal studies into humans, and limitations regarding the chosen animal model, the experimental studies in this thesis are small in size. Into consideration has therefore to be taken the risk for type II error, falsely retaining the null hypothesis. Another limitation to both paper I and paper II is the fact that lung performance was only tested ex vivo, and not in vivo after transplantation. Transplantation in a porcine setup after EVLP has been described in several studies and is feasible, however it is very labour intensive and highly expensive.^{119,138}

A significant limitation to paper II is that in three cases in the Toronto arm EVLP had to be prematurely terminated due to excessive oedema development and lack of perfusate in the circuit. Therefore no data at the second evaluation phase were obtained from these lungs and bias the results of the remaining seven organs to a more favourable outcome, underestimating any differences between the two protocols.

Clinical studies (papers III and IV)

Outcome in patients transplanted after EVLP

The main finding is that the cumulative survival free from re-transplantation, for up to five years was comparable between groups (paper III), in a study from two Scandinavian

centres comparing outcome in 54 patients transplanted with lungs after EVLP with a contemporary control group transplanted with conventional non-EVLP organs. In the EVLP group, PaO₂/FiO₂ at arrival in ICU was significantly lower, and times on ventilator and in ICU were significantly longer, however, time to discharge from hospital, lung function at one year and one-year survival (87% in the EVLP group vs 83% in the conventional group) did not differ between groups.

The high conversion rate (87%, 54 transplantations out of 61 EVLP:s performed) together with good long-term outcome in the EVLP group indicate both that the selection process for which lungs to submit to EVLP was adequate, and that the selection of which lungs to transplant after EVLP was appropriate. In view of an international comparison,^{23,97} high level of donor utilization (27% for conventional lungs), it could be assumed that the threshold for exposing lungs to EVLP is not too low, unnecessarily evaluating organs that could have been transplanted without prior EVLP.

In comparison with previous publications on outcome after transplantation with lungs subjected to EVLP,⁵⁷ our definition on which lungs to submit to EVLP is more strict, and our study group is more homogenous, including only lungs from DBD donors with clear contra-indications to conventional transplantation.

Implementation of an EVLP program together with the application of marginal donor criteria may increase donor utilization to as much as 50%. Centres with an already high acceptance rate may still increase its utilization rate, however the largest potential lies in centres with the lowest utilization rates.

The EVLP program at our two institutions has been previously thoroughly described.^{1,74} It is based on the Lund Protocol,⁴⁰ using a cellular perfusate with banked blood (haematocrit 10-15%), an open left atrium and evaluation at physiological pressures and flow, but with the adaptation of a more restrictive approach to pressure and flow during reconditioning. Decision to accept lungs for transplantation after EVLP is in part based on reaching specific targets in oxygenation capacity of the lungs, and stable hemodynamic and ventilatory parameters. Macroscopic appearance, the look and feel of the organs, and performance during a collapse test are vital parts of the decision. No study has so far presented a validated algorithm for which lungs to accept for transplantation following EVLP.

In this study conversion rate was 87%. A high number, which is in agreement with previous studies,⁶⁵ that may be indicating that we should be even more generous in accepting lungs for EVLP, increasing available organs even further.

Although EVLP according to the Toronto protocol has gained widespread use, and the numbers of EVLP:s using that protocol has surpassed the Lund protocol, this study indicate that good long-term results can be achieved with the Lund protocol. This is in line with our appreciation of EVLP, used primarily as an evaluating and reconditioning tool, although acknowledging the therapeutical potential. Although, time spent on EVLP

is no longer than needed to make a solid decision on the appropriateness of accepting the organs for transplantation.

Inferior short-term results, with a lower PaO₂/FiO₂ at arrival in ICU, longer time on ventilator and longer time in ICU is not a surprise, since only marginal organs were subjected to EVLP, indicating that the selection of organs was appropriate, and that organs were not inappropriately subjected to EVLP. However, since medium- to long-term outcome did not differ between groups, this indicates that residual pathology in transplanted lungs after EVLP was of a reversible nature.

Our times on EVLP are relatively short (median 175 min, range 76-577 minutes). Although it has been stated that EVLP may reduce oedema,⁵⁴ our clinical experience is that any set of lungs may either lose or gain weight during EVLP.^{1,60} Following the publication of a case report on haemofiltration during EVLP,⁶¹ and paper I in this thesis studying this in an experimental setup, we use hemofiltration in clinical practice of all lungs that undergo EVLP.

Outcome measures are dependent not only on the state of the organs accepted after EVLP but on a wide array of factors such as the patient pre-operative status, the diagnosis responsible for the respiratory failure constituting the indication for transplantation, pre- and post-operative use of extracorporeal life support, peri- and postoperative complications such as bleeding, infection and sepsis, and rejection. In paper III, the diagnosis underlying the indication for transplantation were similar between groups, COPD being the most common reason, followed by interstitial lung disease such as fibrosis, being the second most common in both groups, in line with international data.¹³ Bronchiectatic disease, such as cystic fibrosis was more common in the EVLP group, and α -1-anti-trypsin deficiency was more prevalent in the conventional group. Into consideration has also been taken that preoperative mechanical ventilation was more prevalent in the EVLP group (9 vs. 4 %) while preoperative ECMO was more prevalent in the conventional group (2 vs. 6 %).

Time to extubation was more than doubled in the EVLP group compared to control, with a median of 18 compared to 7 hours. The range is substantial, varying from 2 to 2280 hours in the whole cohort. This difference between groups was highly significant. Although, in both groups, time to extubation was low in comparison with earlier publications, Toronto reporting a median time to extubation of 2 days (range 1 -101),⁵⁷ Aigner et al. reporting median time of 48 hours and Zych et al. reporting median times of 214 hours.^{87,89} This is in line with the focus at our two centres on early extubation, to non-invasive ventilatory support if necessary, promoting early mobilisation of the patient, aiming to avoid complications related to invasive ventilation.

Time in intensive care is also a multifactorial outcome measure, depending on the preoperative state of the patient. Any pre-operative extracorporeal support is an indicator of poor pre-operative status of the patient, most often leading to a more prolonged course in intensive care. Any intra- or postoperative complication, such as bleeding,

postoperative respiratory failure and ventilator-associated pneumonia or sepsis necessitating prolonged invasive ventilatory support may extend times spent in ICU substantially. This is reflected in paper III by the fact that although median stay is only 3 days in the control group and 4 days in the EVLP group, range is between 1 and 156 days. Different indications and organisations for intensive care versus care in step-down or high-dependency units may make comparison between different studies troublesome. The results of paper III is well in line with other larger materials published, Cypel et al. reporting a median ICU stay of 4 days and Aigner et. al of 5.5 days.^{57,87} In all those studies there was a substantial range in ICU stay.

Variables during EVLP predicting good organ function in the donor

The decision to proceed with transplantation after EVLP is multifactorial, integrating knowledge about the donor, the static and dynamic performance of the lungs during EVLP with regards to blood gas analysis, vascular resistance and lung dynamics, as well as the touch, feel and look of the organs. No single factor universally singles out the good from the bad organs, and to date no validated protocol regarding which organs to accept has been presented.

It could be assumed that the better the organs perform during EVLP, the better they will perform in vivo after transplantation. However, we could not in this study find any correlations between physiologic variables measured during EVLP and three commonly used outcome measures in the transplanted patient. It might be explained by selection bias, as only lungs regarded as suitable for transplantation were used, making numbers too small in this study to detect any correlations that actually exist.

Although research is active in finding both novel physiological parameters better reflecting suitability for transplantation, such as the PF-difference, and biochemical markers for good transplant function, the decision process during EVLP will likely continue to be a synthesis of multiple factors.

Limitations

The clinical studies are retrospective studies based on prospectively collected data. Although the same EVLP protocol has been used at our two centres, minor differences exist in the selection processes and in the clinical perioperative management of patients. The surgical method also differs between centres, sequential bilateral thoracotomy without the use of ECC being favoured in Gothenburg, while the use of ECC and sternotomy is more prevalent in Copenhagen, during the study period.

Although being one of the larger materials presented to date, numbers are still small, and conclusions from this non-randomized study cannot be compared with that of a randomized one. Numbers at risk at the far end of the survival analysis are small and care has to be taken in interpreting differences between groups.

There were missing registry data in certain variables, and although great efforts were made to make the data as complete as possible, this could possibly have influenced results.

In paper IV, there has already been a selection process, in that only lungs accepted for transplantation are included in the analysis. They are all considered acceptable for transplantation, fulfilling criteria, and it could therefore be that numbers are too small in this study to detect any existing correlations between variables during EVLP and outcome. The outcome parameters, in addition, are affected not only by the status of the transplanted organ, but by the preoperative state of the recipient and any post-operative complications such as sepsis, pneumonia or rejection.

In paper IV, quite a few parameters are investigated for correlations with the outcome parameters. Had there been positive correlations found, in the setting of analysing that many parameters, it might have been due to chance alone (type I error).

CONCLUSIONS

In paper I, after induction of pulmonary oedema, haemofiltration during EVLP increased perfusate oncotic pressure, decreased lung weight, had beneficial effects on compliance and the potential to reduce pulmonary oedema content by as much as 50%. It did however not improve lung oxygenation capacity.

EVLP alone, without haemofiltration, did not reduce the degree of pulmonary oedema in this model. Perfusate oncotic pressure increased during EVLP in the control group, contradicting the practice of intermittent perfusate exchange during EVLP.

In the EVLP comparison model (paper II), keeping in mind that three animals in the ACA group were terminated prematurely due to significant oedema formation and inability to continue EVLP, lung oedema formation and decreasing lung compliance during EVLP occurred similarly with both techniques of EVLP. There were no significant differences between them with regards to lung function, inflammatory response, ischemia/reperfusion injury or histopathological changes.

In the combined Copenhagen and Gothenburg clinical study (paper III), one of the larger studies of outcome after EVLP in DBD donors, time on ventilator and time in ICU after lung transplantation was significantly longer in the EVLP group. However, time to discharge, lung function at one year and survival in patients transplanted with lungs evaluated with EVLP did not differ significantly from patients transplanted with conventional donor lungs.

In an attempt to identify risk factors during EVLP for poor outcome after lung transplantation (paper IV), we demonstrated no correlations between commonly measured variables during EVLP and short-term outcome.

FUTURE PERSPECTIVES

EVLP as a means to evaluate marginal lungs is by now well established in clinical practice. The decision of accepting organs is moved from unfamiliar surroundings at the donor hospital to the home clinic, where decisions can be made in a controlled fashion after thorough evaluation. However, initiating an EVLP program is a substantial undertaking, and there has been an increasing interest in the concept of centralized EVLP centres, primarily in the USA. Since prolonged preservation times in conjunction with EVLP has been shown to be well tolerated, the extra time added to the transplantation process by such an arrangement, is considered acceptable. The basic idea is to provide EVLP services to transplant centres without their own expertise in the field, retrieving the lungs, performing EVLP and thereafter forwarding the approved organs to their respective transplantation centre. In this way, EVLP could be made more generally available, and benefit drawn from expertise gained at such high-volume centres, increasing quality and enabling research. There is of course also a commercial incentive, especially in the USA, in providing these services. Regulatory and process issues remain to be solved.

As previously described, there is interest in finding reliable biomarkers to predict lung function after transplantation. None has so far reached clinical use, and the relatively limited time frame necessitating rapid analysis and test results of any potential biomarker is an obstacle that has to be overcome.

Contemporary methods of evaluating lungs during EVLP mirror the overall performance of the organs. However, in many instances only a lobe or one lung may be pathological. Imaging modalities, such as real time CT, during EVLP might add to decision-making in the future.

In line with the insights into the importance of a lung protective strategy during ventilation of patients in the ICU, there have been experimental studies reporting positive effects of certain ventilation strategies during EVLP. Further interest in this field and studies adapting the ventilation strategies used during clinical EVLP can be expected.

Although the $\text{PaO}_2/\text{FiO}_2$ ratio has been and still is an important factor in assessing lung function, it is by now well known that it is a rather blunt tool, especially when using acellular perfusate. Focus will likely shift further to other parameters better and more reliably predicting outcome. Compliance is one parameter that has been attributed a greater significance lately. One emerging example is the PF-difference (the difference between PF-ratios at different fractions of inspired oxygen) mirroring the intrapulmonary shunt fraction, having been correlated with short-term outcome.

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APPENDIX (PAPERS I-IV)

