Identification of regional variation in gene expression and inflammatory proteins in donor lung tissue and ex vivo lung perfusate

Identification of regional variation in gene expression and inflammatory proteins in donor lung tissue and ex vivo lung perfusate

Bonnie T. Chao[1,2], Andrew T. Sage[1,3], PhD, Jonathan C. Yeung, MD PhD[1,3,5,6], Xiaohui Bai, MSc[1], Jin Ma, PhD[4], Tereza Martinu, MD MHSc[1,3,6,7], Mingyao Liu, MD[1,3,5,6,7], Marcelo Cypel, MD[1,3,5,6], Dirk Van Raemdonck, MD PhD[18,9], Laurens J. Ceulemans, MD[8,9], Arne Neyrinck, MD PhD[110,11], Stijn Verleden, PhD[8,12], Shaf Keshavjee, MD[1-3,5,6]

1. Toronto Lung Transplant Program and Latner Thoracic Research Laboratories, Toronto General Hospital Research Institute, University Health Network, Toronto, Ontario, Canada
2. Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada
3. Ajmera Transplant Centre, University Health Network, University of Toronto, Toronto, Ontario, Canada
4. Biostatistics Research Unit, University Health Network, Toronto, Ontario, Canada
5. Department of Surgery, Temerty Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
6. Institute of Medical Science, Temerty Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
7. Department of Medicine, Temerty Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada
8. BREATHE, Department of CHROMETA, KU Leuven, Leuven, Belgium
9. Department of Thoracic Surgery, University Hospitals Leuven, Leuven, Belgium
10. Department of Cardiovascular Sciences, KU Leuven, Leuven, Belgium
11. Department of Anesthesiology, University Hospitals Leuven, Belgium
12. Department of ASTARC, University of Antwerp, Belgium

Running title: Consistency of Inflammatory Biomarkers in Lung Biospecimens

Disclosure Statement

SK serves as Chief Medical Officer of Traferox Technologies and as a Consultant to Lung Bioengineering, SQI Diagnostics, and receives research support from United Therapeutics and CSL Behring, outside the submitted work. MC is a Consultant for Traferox Technologies and Lung Bioengineering. The authors fully adhere to policies at University Health Network that ensure academic integrity and management of potential conflicts of interest between authors and industry partners. All other authors have no conflict of interest to declare for the study results.

Acknowledgments

The authors wish to thank the Toronto Lung Transplant Program Biobank team and KU Leuven team for their efforts to collect and process the samples used in this study. Additional thanks to the Organ Perfusion Specialists on the EVLP Team and Rasheed Ghany for their help with the EVLP sampling and clinical database used in this study, respectively, and to Jerome Valero for manuscript review.

Funding Statement: N.A.

Corresponding Author Contact Information
Shaf Keshavjee MD
Director, Toronto Lung Transplant Program
Division of Thoracic Surgery
Toronto General Hospital, University Health Network
200 Elizabeth St. 9N-946
Toronto, ON, Canada M5G 2C4
Tel: 416 340 4010
Email: shaf.keshavjee@uhn.ca

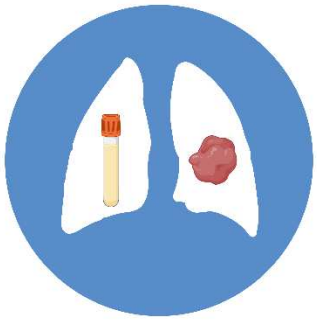
Article Word Count

250 (abstract) + 2930 (main text)

Glossary of Abbreviations:

coefficient of variance (CV), ex vivo lung perfusion (EVLP), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), left lower lobe (LLL), left upper lobe (LUL), right lower lobe (RLL), right upper lobe (RUL)

Central Picture + Legend (74/90 characters including spaces)



Sample validity of tissue biopsy and ex vivo lung perfusate in donor lungs

Central Message (189/200 characters including spaces)

Cytokine gene and protein expressions from biopsies and ex vivo lung perfusates are representative of the lung. Caution is advised when sampling from the lingula or lungs with focal injury.

Perspective Statement (351/405 characters including spaces)

In clinical diagnostics, whether a sample is truly representative of the organ has been a longstanding controversy. In this study, we examine inflammatory profiles using biopsies and ex vivo perfusates from various donor lungs, thus providing valuable insights to the sample validity of gene and protein measurements in clinical donor lung evaluation.

Structured Abstract (250/250 words)

Objective: Diagnosing lung injury is a challenge in lung transplantation. It has been unclear if a single biopsy specimen is truly representative of the entire organ. Our objective was to investigate the human lung inflammatory biomarkers using lung tissue biopsies and ex vivo lung perfusion (EVLP) perfusate.

Methods: Eight human donor lungs declined for transplantation were air-inflated, flash frozen, and partitioned from apex to base. Biopsies were then sampled throughout the lung. Perfusate was sampled from four lung lobes in eight additional donor lungs subjected to EVLP. The levels of IL-6, IL-8, IL-10, and IL-1β were measured using qRT-PCR from lung biopsies, and ELISA from EVLP perfusate.

Results: The median intra-biopsy equal-variance p-value was 0.50 for mRNA biomarkers in tissue biopsies. The median intra-biopsy coefficient of variance (CV) was 18%. In donors with no

apparent focal injuries, the biopsies in each donor showed no difference in various lung slices, with a CV of 20%. The exception was biopsies from the lingula and injured focal areas that demonstrated larger differences. Cytokines in EVLP perfusate showed minimal variation among different lobes (CV = 4.9%).

Conclusions: Cytokine gene expression in lung biopsies was consistent and the biopsy analysis indeed reflects the whole lung, except when specimens were collected from the lingula or an area of focal injury. EVLP perfusate also provides a representative measurement of lung inflammation from the draining lobe. These results will reassure clinicians that a lung biopsy or an EVLP perfusate sample can be used to inform donor lung selection.

Key words (3-7 keywords)**:** tissue sampling, gene expression profiling, inflammatory cytokines, ex vivo lung perfusion

Main Text (3255/3500 words)

Introduction

Inflammatory cytokines are an important family of biomarkers known to reflect donor lung injury during transplantation[1]. The significance of understanding cytokine-derived lung inflammation has been demonstrated at both the nucleic acid[1–3] and protein[4–8] levels. However, in these studies, lung biopsies are generally collected from a single location, and perfusate samples are usually collected from a single channel of an ex vivo lung perfusion (EVLP) circuit. It remains unclear whether these approaches truly reflect the inflammatory response across the entire organ, as donor lung injury is thought to be heterogeneous in the lung. This raises an important question regarding sampling reliability: does a small biopsy of lung tissue or single perfusate sample reflect the status of the whole lung for biological assessment?

Several studies have shown that cytokines, such as interleukin-6 (IL-6), IL-8 or CXCL-8, IL-10, and IL-1β indicate the severity of inflammatory responses and can be used to determine transplant suitability[1,3–6,8]. Pre- and post-transplant cytokine and chemokine levels of IL-6, IL-8, IL-10, and IL-β in plasma, EVLP perfusate, and tissue have been reported to be predictive of development of patient outcome – particularly primary graft dysfunction, a condition which contributes to the development of chronic lung allograft dysfunction[1,3,8–12]. While the role of these inflammatory markers in lung transplantation has been well-established, a systematic evaluation of the techniques used to collect donor lung biospecimens and study these biomarkers has not yet been conducted to date.

Herein, we provide a quantitative investigation of the regional variability in the lung of different sampling techniques and locations to determine if they are indeed reflective of the biologic status of the whole lung (Figure 1). Protein and mRNA analysis of cytokines were studied in two sample types—EVLP perfusate and tissue biopsies. In the following sections, the sampling method and signal variability will be discussed, and the results of this study will be used to provide insights into the reliability of sampling perfusates and tissue biopsies in lung transplantation.

Material and Methods

Study Group

In both tissue biopsy and perfusate analyses described below, samples were prospectively collected at both institutions and then retrospectively analyzed.

Tissue Biopsy Analysis: Eight human donor lungs that were declined and not used for clinical transplantation at University Hospitals Leuven Lung Transplant Program (Leuven, Belgium) from 2013 to 2016 were studied. Written informed consent of donors was not obtained, since local Belgian legislation stipulates that donor lungs considered to be of insufficient quality for transplantation can be offered for research (Ethics Committee University Hospitals Leuven, Belgium, Institutional Review Board number S52174, S61653). Anonymized donor demographics were collected via the local transplant coordination office and Eurotransplant.

Whole lungs were inflated with air and flash frozen in liquid nitrogen vapor. A mix of left and right lungs were sliced into ten to fourteen 1 cm sections from the apex to the base (Figure 2A). A

similar sampling procedure is described in a previous study[13,14]. Five biopsies 1 cm^3 in size were sampled from cores taken from the lung sections for analysis, with biopsies representative of anterior and posterior positions (approximately 2:1 ratio). Three of the five biopsies were sampled from the same slices across eight donor lungs, specifically slices #3, 6, and 9. Another biopsy was sampled from a site of visually apparent focal injury based on macroscopic examination of the lung slices, if one existed, and the final biopsy from each lung was sampled to best represent the lingula. Each 1 cm^3 biopsy was divided into three smaller pieces and these samples were analyzed as biopsy replicates. The sampling schematic is shown in Figure 2A. In total, there were 3 samples from each biopsy, 5 biopsies from each subject, and 8 subjects, yielding n=120 samples altogether.

EVLP Perfusate Analysis: Eight randomly selected donor lungs that were clinically assessed on EVLP at Toronto General Hospital, University Health Network (UHN, Toronto, Canada) between 2018 and 2019 were studied. All samples and clinical information were collected with patient consent, and the study was approved by the UHN Research Ethics Board (REB#12-5488).

The Toronto EVLP Technique has been previously described[15]. Briefly, donor lungs were placed on the EVLP circuit and perfused for three to six hours. Perfusate samples from the right upper lobe (RUL), right lower lobe (RLL), left upper lobe (LUL), and left lower lobe (LLL) of each donor lung were directly sampled from the respective pulmonary veins using a syringe with a 25-gauge needle (Figure 2B). All perfusates were collected at the second hour after the start of perfusion, except for Case #9 which was drawn at the third hour, and Case #11 which was drawn at the first hour.

Biomarker Quantification

Gene expression assay: mRNA expression levels of IL-6, IL-8, IL-10, and IL-1β were measured in tissue biopsies using qRT-PCR. RNA was extracted from the tissue using the RNeasy Micro Kit (Qiagen Ltd, Hilden, Germany). cDNA was synthesized with a reverse transcriptase kit (Thermo Fisher Scientific, Waltham, MA) and measured using quantitative polymerase chain reaction (qPCR) (Thermo Fisher Scientific). A calibration curve was generated using six standard points, and the initial mRNA level of each cytokine was interpolated from the standard curve. Importin 8 (IPO8) and glucuronidase beta (GUSB) were used as housekeeping genes, and the relative mRNA expressions of IL-6, IL-8, IL-10, and IL-1β were calculated by normalizing the mRNA expression to the geomean of IPO8 and GUSB by calculating the ratio. The primer sequences are included in Table S1.

Protein Assay: EVLP perfusate samples were tested immediately after collection using an ELLA multiplex assay (Protein Simple, San Jose, CA), according to the manufacturer's instructions. Unprocessed perfusate samples were loaded undiluted for IL-1β and IL-10 and diluted 50X for IL-6 and IL-8 with sample diluent. Three technical replicates for IL-6, IL-8, IL-10, and IL-1β were recorded as part of the analyses.

Statistical Analysis

Python and John's Macintosh Project from Statistical Analysis System (JMP Software, Cary, NC) were used.

Tissue Biopsy Analysis: To investigate the intra-biopsy variance of the three samples within a given biopsy, the Brown-Forsythe equal-variance test was used. For the intra-lung analysis, One-way ANOVA was conducted to compare the five biopsies within each case. One single ANOVA

technique was applied to analyze cytokine gene expressions from each donor and cytokine, and the same ANOVA technique was repeated for each donor and cytokine, as shown in Figure S1. The Tukey-Kramer method was used to compare pairs of biopsy means in each case. For each cytokine, the intra-biopsy coefficient of variance percentages (%CV) was calculated for each biopsy. The intra-lung %CV was then calculated for three groups: 1) slices #3, 6, and 9; 2) slices #3, 6, and 9 plus the injury biopsy; and 3) slices #3, 6, and 9 plus the lingula. The % CVs were then compared using the Mann-Whitney U test.

EVLP Perfusate Analysis: CVs were calculated to quantify the differences in IL-6, IL-8, IL-10, and IL-1β levels between RUL, RLL, LUL, and LLL.

Results

Tissue mRNA Analysis: To evaluate the variation in lung tissue sampling, we first investigated the profile of inflammatory cytokine mRNA expressions in lung biopsies. Table 1 shows a summary of donor lung characteristics, including the reason for the lung being declined for clinical transplantation (Case 1-8).

Inflammatory gene expression in lung biopsies was tested using quantitative real time polymerase chain reaction (qRT-PCR) for the 4 cytokines for each biopsy specimen measured in triplicate. Figure 3 illustrates a swarm plot of each individual measurement demonstrating that inter-lung variations were of the same magnitude as intra-lung variations.

Within each case, the intra-biopsy variances of each cytokine from the five biopsies were compared using the Brown-Forsythe equal-variance test. The median p-values were 0.51, 0.55, 0.58, and 0.29 for IL-6, IL-8, IL-10, and IL-1β, respectively, indicating that the intra-biopsy variances were not significantly different. The median intra-biopsy CV across all eight subjects and four cytokine genes was 18.4% [12.3% – 31.8%].

ANOVA and pairwise mean comparisons across the five biopsies of each donor are summarized in Figure S1, where cases are presented in rows and cytokines in columns. In each table of a specific case and cytokine, biopsy locations in rows were compared with those in columns, and any significant differences were denoted with coloured grids. Generally, IL-6, IL-8, IL-10, and IL-1β gene expressions tended to be similar across biopsies 3, 6, and 9. When significant differences between biopsies were observed, it was in the lingula or focal injury area (Figure S1).

Cytokine gene expression values were then pooled to investigate global variance across lung biopsies. Figure S2 shows a normalized box plot of the five biopsy types across all subjects and cytokines. Each datapoint was derived by subtracting the mean of slices #3, 6, and 9 from the same subject and cytokine, and then dividing by the standard deviation of the same group. The normalized data of all subjects and cytokines were used to show the relative cytokine levels of the five biopsies (Figure S2A). Consistent with the findings in Figure S1, both the focal injury areas ($p=0.0021$) and lingular ($p=0.0002$) biopsies exhibited significant differences from slices #3, 6, and 9 (Figure S2A), but the overall cytokine gene expression profile of the injury and lingula biopsies was equivalent to each other (Figure S2A, $p=0.50$). When separated by cytokine (Figure S2B-E), IL-6 and IL-8 showed no significant differences between slices #3, 6, 9 and the lingula or injury biopsies ($p>0.05$, Figure S2B, C). There was significantly higher IL-10 gene expression in

237 the injury sites (Figure S2D), whereas the lingula showed a significantly higher expression of IL-
238 1β (Figure S2E).

239 We next investigated the presence of any global differences in the variances of gene expression
240 for lungs rejected for graft or non-graft related reasons. Table S2 shows that the median CVs of
241 slices #3, 6, and 9 for donor lungs declined for non-graft related reasons (Cases 2, 3, and 8) was
242 20.3%, which did not significantly differ from the intra-biopsy CV (p=0.80). Compared to lungs
243 rejected for graft-related reasons (Cases 1, 4-7), lungs rejected for non-graft related reasons
244 showed a significantly lower global intra-lung variance in inflammatory gene expression (Table 2,
245 20.3% vs. 47.4%, p=0.0017). Regardless of the reason for rejection, the variance in gene
246 expression was approximately 50% when either the lingula or injury biopsy were added to slices
247 #3, 6, 9 (Table 2).

248 In donor lungs rejected for non-graft related reasons, when the lingula or the injury area biopsy
249 was added to slices #3, 6, and 9, there was a significant 2.5-fold increase in the variation compared
250 to slices #3, 6, and 9 alone (Table 2, p=0.026 (lingula), 0.019 (injury)). The variations of gene
251 expressions in biopsies significantly increased with the addition of injury and lingula biopsies. For
252 donor lungs rejected for graft-related reasons, the CV of slices #3, 6, and 9 had a non-significant
253 increase from 47.4% to 50.7% and 57.3%, respectively, when the lingula or injury biopsy was
254 included (Table 2). Although the overall signal variations were high, there were no significant
255 changes in variations among different biopsy sites.

256 *Perfusate Protein Analysis:* Protein levels of inflammatory cytokines in EVLP perfusates were
257 examined to validate the nucleic acid findings. Table 3 contains a summary of donor lungs that
258 underwent ex vivo assessment (Cases 9-16). Figure 4 shows a swarm plot of the individual

cytokine replicates from different lung lobes measured using ELISA. Each color represents a particular lobe.

The cytokine levels in EVLP perfusate were consistent across the four lobar samples (RUL, RLL, LUL, and LLL; Figure 4). The mean CVs from the four lung regions within each donor were 5.6 ± 3.5% for IL-6, 7.0 ± 2.6% for IL-8, 5.0 ± 1.9% for IL-10, and 6.5 ± 2.7% for IL-1β (Table S2). The reference, intra-assay CV values reported by the manufacturer were: 2.4% to 3.9% for IL-6, 4.9% to 6.6% for IL-8, 4.6% to 6.0% for IL-10, and 3.7% to 4.9% for IL-1β (Table S2). Among the eight donor lungs with various injury statuses according to the lung oxygenation and EVLP outcome, lobar cytokine levels and changes in lobar cytokine levels did not vary according to different injury levels.

Discussion

In this study, IL-6, IL-8, IL-10, and IL-1β measurements in donor lungs were evaluated using two different biospecimen sampling techniques—tissue biopsies and EVLP perfusate sampling. In the assessment of mRNA expression, five tissue biopsies were sampled from each lung, which included three random biopsies, one biopsy from the lingula and one from a noted focal injury site, if present. IL-8 and IL-10 were found to be elevated in biopsies with focal injury. IL-8 is widely found to be pro-inflammatory, while the anti-inflammatory effect of IL-10 can contribute to tissue protection and be elevated in the presence of lung injury. IL-10 is often observed to be co up-regulated with other pro-inflammatory cytokines such as IL-1β and TNF-α[16]. Regional EVLP perfusate samples for each of the lung lobes were additionally analysed for cytokine protein levels.

Overall, we demonstrated that cytokine mRNA expression levels throughout the human donor lung are relatively comparable. That is, *a random biopsy taken from a donor lung is indeed representative of the whole lung*. Exceptions were areas of the donor lungs that had a visually (macroscopically) obvious focal injury or tissue biopsies collected from the lingula. The lingula is known to represent a region in the lung with greater variability[17–20] that generally should be avoided as a site for biopsy, as it is more likely to be diseased and less likely to be representative of the whole lung. Inter-lung variations in inflammatory gene expression were at least on par with, if not larger than intra-lung variations for all lungs. As expected, donor lung areas with obvious injury showed higher variability at the gene level, although no significant differences in signal variations were found between biopsy sites. Notably, cytokine proteins in perfusate samples collected during EVLP were very similar across different lung lobes. Therefore, *perfusate samples collected from the EVLP circuit are a reliable and representative of the donor lung.*

The measurement deviations of the present study were within the acceptable error ranges in biomarker quantitation of many industrial platforms, especially given that technical errors are inevitably common in RNA extraction and qPCR[21–23]. The measurement variability was also consistently smaller within-biopsy than across-biopsy, indicating that tissues sampled closer together yielded more similar mRNA expression. Importantly, this study confirms that banked donor lung tissue biopsies and EVLP perfusates are suitable for biomarker studies in that they do reflect the status of the whole lung. In donor lungs without gross focal injury, biopsies collected at random sites throughout the lung had cytokine mRNA expression with variations similar to intra-biopsy variations. In other words, sampling and testing tissue biopsies from different locations yielded similar variances to repeatedly measuring the same biopsy. The lack of an apparent focal injury resulted in a more homogeneous representation of the overall lung state. In contrast, donor

lungs with specific visible gross injuries exhibited heterogeneity in the lungs and different degrees of inflammation. Indeed, donor lungs that are potentially injured and marginally acceptable are more likely to be heterogeneous because infection, atelectasis and aspiration often occur in the lower lobes, while emphysema is found more often in the upper lobes[24]. In a prior histologic study, the authors observed high inter- and intra-lobar variations in twenty morphologic variables[24]. In this study, we confirmed a higher degree of variability in inflammatory cytokines in these lungs. Sampling across lungs with gross focal injuries may be valid with equal amounts of variations, but due to the inherently higher variances in gene expressions, more than one biopsy may be required to discern the effect size with sufficient power.

Throughout various analyses, the lingular biopsies contributed to significant variations in biopsy mRNA expressions. This occurred in all eight subjects regardless of the status of organ injury and points to the intrinsic characteristics of the lingula rather than the discrepancies between different donors. Several previous reports have also identified concerns with the lingula showing higher involvement of diseases[17–20,26,27]. Existing literature that compared the lingula to other biopsies focused on the histopathological technique[28–31]. Many have suggested that the anatomical features of the lingula act as an isolated sink for chronic inflammation and scarring, with non-specific injury presentation unrelated to associated diffuse lung diseases[17–20]. Deep fissures in the lingula result in scanty parenchymal bridges, effectively blocking collateral ventilation[14]. In addition, drainage of mucous is difficult in the lingula due to the long and narrow lingular segmental bronchus along with the acute take-off angle. This peripheral clogging of the small airways, combined with the predisposition of mucus accumulation, result in passive congestion in the lingula and can often lead to persistent atelectasis and inflammation without underlying lung disease[19,20]. The RML shares a few anatomical similarities with the lingula; both have long and narrow bronchi leading

to mucus retention, inefficient collateral ventilation, and inflammation. As a result, the RML may exhibit a similar biomarker profile as the lingula, but more studies are needed to confirm this statement.

Sampling of cytokines from EVLP perfusate of different lung lobes yielded very small variations. Because perfusates in EVLP are continuously being mixed and redistributed in different lobes, the transit time in which the perfusate flows through the lungs may not be long enough to result in critical differences in cytokine levels. While this may pose challenges in discerning local injuries in donor lungs, other methods such as histology and X-ray imaging could reveal any potential regional concerns. Alternatively, the circulating cytokines may influence inflammatory responses among different cell types through cytokine receptors that are widely distributed in different cell types in the lung. This may explain why anti-inflammatory therapeutics, such as IL-10 gene therapy[32] or alpha-1 antitrypsin[33, 34], can inhibit inflammatory responses during EVLP.

For studies involving biospecimen collection to assess the donor lung for lung transplantation, the following recommendations are supported by this study: (i) a random biopsy of the lung is reflective of the overall status of the donor lung; (ii) when there is an obvious focal lung injury, collection of a biopsy of that specific site will yield important information related to that specific injury; (iii) biopsy of the lingula should be avoided if possible due to high signal variability and intrinsic chronic inflammation; and (iv) collection of a single perfusate sample during EVLP provides a uniform picture reflective of the inflammatory state of the whole organ. One limitation of this study is the small donor pool in the biopsy cohort. It is possible that with eight donor lungs, the statistical power was insufficient to detect small differences between biopsy locations. Future

studies can expand the number of donor lungs to include a wider range of injury statuses and discern any potential differences between biopsy locations with a higher statistical power.

In this present study, we confirm that a random biopsy taken from the donor lung can be expected to be representative of the whole donor lung state. Additionally, assay of inflammatory cytokines in the ex vivo lung perfusate is representative of the donor lung. Taken together these results will reassure clinicians that a biopsy of the donor lung or a sample of EVLP perfusate are indeed representative of the whole lung being examined and can be used to aid diagnosis and guide therapy. This information is particularly important and relevant as we and others continue to interrogate lung biospecimens towards the development of novel assays to diagnose injuries, predict outcomes and develop targeted treatments to heal and repair injured donor lungs for successful clinical transplantation.

References (34/35)

1. Kaneda H, Waddell TK, De Perrot M, et al. Pre-Implantation Multiple Cytokine mRNA Expression Analysis of Donor Lung Grafts Predicts Survival After Lung Transplantation in Humans: **Donor Multiple Cytokine Analysis Predicts Survival**. *American Journal of Transplantation*. 2006;6(3):544-551. doi:10.1111/j.1600-6143.2005.01204.x

2. Matilla JM, García-Yuste M, Crespo MS, et al. Interleukin-8 Expression in Lung Tissue During Ischemia-Reperfusion. *Archivos de Bronconeumología ((English Edition))*. 2007;43(10):542-548. doi:10.1016/S1579-2129(07)60125-6

3. Saito T, Takahashi H, Kaneda H, et al. Impact of Cytokine Expression in the Pre-Implanted Donor Lung on the Development of Chronic Lung Allograft Dysfunction Subtypes: Cytokines in Pre-Implant Donor Lung and CLAD. *American Journal of Transplantation*. 2013;13(12):3192-3201. doi:10.1111/ajt.12492

4. De Perrot M, Sekine Y, Fischer S, et al. Interleukin-8 Release during Early Reperfusion Predicts Graft Function in Human Lung Transplantation. *Am J Respir Crit Care Med*. 2002;165(2):211-215. doi:10.1164/ajrccm.165.2.2011151

5. Andreasson ASI, Karamanou DM, Gillespie CS, et al. Profiling inflammation and tissue injury markers in perfusate and bronchoalveolar lavage fluid during human *ex vivo* lung perfusion. *Eur J Cardiothorac Surg*. Published online January 12, 2017:ezw358. doi:10.1093/ejcts/ezw358

6. Andreasson ASI, Borthwick LA, Gillespie C, et al. The role of interleukin-1β as a predictive biomarker and potential therapeutic target during clinical ex vivo lung perfusion. *The Journal of Heart and Lung Transplantation*. 2017;36(9):985-995. doi:10.1016/j.healun.2017.05.012

7. Sage AT, Bai X, Cypel M, Liu M, Keshavjee S, Kelley SO. Using the inherent chemistry of the endothelin-1 peptide to develop a rapid assay for pre-transplant donor lung assessment. *Analyst*. 2015;140(24):8092-8096. doi:10.1039/C5AN01536G

8. Sage AT, Richard-Greenblatt M, Zhong K, et al. Prediction of donor related lung injury in clinical lung transplantation using a validated ex vivo lung perfusion inflammation score. *The Journal of Heart and Lung Transplantation*. Published online March 2021:S1053249821022191. doi:10.1016/j.healun.2021.03.002

9. Allen JG, Lee MT, Weiss ES, Arnaoutakis GJ, Shah AS, Detrick B. Preoperative Recipient Cytokine Levels Are Associated With Early Lung Allograft Dysfunction. *The Annals of Thoracic Surgery*. 2012;93(6):1843-1849. doi:10.1016/j.athoracsur.2012.02.041

10. Hoffman SA, Wang L, Shah CV, et al. Plasma Cytokines and Chemokines in Primary Graft Dysfunction Post-Lung Transplantation. *American Journal of Transplantation*. 2009;9(2):389-396. doi:10.1111/j.1600-6143.2008.02497.x

11. Verleden SE, Martens A, Ordies S, et al. Immediate post-operative broncho-alveolar lavage IL-6 and IL-8 are associated with early outcomes after lung transplantation. *Clin Transplant*. 2018;32(4):e13219. doi:10.1111/ctr.13219

12. Calfee CS, Budev MM, Matthay MA, et al. Plasma Receptor for Advanced Glycation End-products Predicts Duration of ICU Stay and Mechanical Ventilation in Patients After Lung Transplantation. *The Journal of Heart and Lung Transplantation*. 2007;26(7):675-680. doi:10.1016/j.healun.2007.04.002

13. Vanstapel A, Dubbeldam A, Weynand B, et al. Histopathologic and radiologic assessment of nontransplanted donor lungs. *Am J Transplant*. 2020;20(6):1712-1719. doi:10.1111/ajt.15790

14. Verleden SE, Kirby M, Everaerts S, et al. Small airway loss in the physiologically ageing lung: a cross-sectional study in unused donor lungs. *The Lancet Respiratory Medicine*. 2021;9(2):167-174. doi:10.1016/S2213-2600(20)30324-6

15. Cypel M, Yeung JC, Hirayama S, et al. Technique for Prolonged Normothermic Ex Vivo Lung Perfusion. *The Journal of Heart and Lung Transplantation*. 2008;27(12):7.

16. Clarke CJP, Hales A, Hunt A, Foxwell BMJ. IL-10-mediated suppression of TNF-α production is independent of its ability to inhibit NFκB activity. *Eur J Immunol*. 1998;28(5):1719-1726. doi:10.1002/(SICI)1521-4141(199805)28:05<1719::AID-IMMU1719>3.0.CO;2-Q

17. Gaensler EA, Carrington CB. Open Biopsy for Chronic Diffuse Infiltrative Lung Disease: Clinical, Roentgenographic, and Physiological Correlations in 502 Patients. *The Annals of Thoracic Surgery*. 1980;30(5):411-426. doi:10.1016/S0003-4975(10)61291-X

18. Ray JF, Lawton BR, Myers WO, et al. Open Pulmonary Biopsy. *Chest*. 1976;69(1):43-47. doi:10.1378/chest.69.1.43

19. Gudbjartsson T, Gudmundsson G. Middle Lobe Syndrome: A Review of Clinicopathological Features, Diagnosis and Treatment. *Respiration*. 2012;84(1):80-86. doi:10.1159/000336238

20. Ayed AK. Resection of the Right Middle Lobe and Lingula in Children for Middle Lobe/Lingula Syndrome. *Chest*. 2004;125(1):38-42. doi:10.1378/chest.125.1.38

21. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The Ultimate qPCR Experiment: Producing Publication Quality, Reproducible Data the First Time. *Trends in Biotechnology*. 2019;37(7):761-774. doi:10.1016/j.tibtech.2018.12.002

22. Peccoud J, Jacob C. Theoretical uncertainty of measurements using quantitative polymerase chain reaction. *Biophysical Journal*. 1996;71(1):101-108. doi:10.1016/S0006-3495(96)79205-6

23. Moignard V, Göttgens B. Transcriptional mechanisms of cell fate decisions revealed by single cell expression profiling. *BioEssays*. 2014;36(4):419-426. doi:10.1002/bies.201300102

24. Niikawa H, Okamoto T, Ayyat KS, et al. A novel concept for evaluation of pulmonary function utilizing PaO2/FiO2 difference at the distinctive FiO2 in cellular *ex vivo* lung perfusion—an experimental study. *Transpl Int*. Published online April 15, 2019:tri.13426. doi:10.1111/tri.13426

25. Winterbauer RH, Hammar SP, Hallman KO, et al. Diffuse interstitial pneumonitis. *The American Journal of Medicine*. 1978;65(4):661-672. doi:10.1016/0002-9343(78)90855-0

26. Spielberg DR, Brody AS, Baker ML, Woods JC, Towe CT. Ground-glass burden as a biomarker in neuroendocrine cell hyperplasia of infancy. *Pediatric Pulmonology*. 2019;54(6):822-827. doi:10.1002/ppul.24301

27. Hillerdal G. Rounded Atelectasis. *Chest*. 1989;95(4):836-841. doi:10.1378/chest.95.4.836

28. Miller RR, Nelems B, Muller NL, Evans KG, Ostrow DN. Abstract - Lingular and right middle lobe biopsy in the assessment of diffuse lung disease.html. Published online September 1987. https://pubmed.ncbi.nlm.nih.gov/3632113/

29. Temes RT, Joste NE, Allen NL, Crowell RE, Dox HA, Wernly JA. The lingula is an appropriate site for lung biopsy. *The Annals of Thoracic Surgery*. 2000;69(4):1016-1018. doi:10.1016/S0003-4975(99)01565-9

30. Wetstein L. Sensitivity and Specificity of Lingular Segmental Biopsies of the Lung. *Chest*. 1986;90(3):383-386. doi:10.1378/chest.90.3.383

31. Newman SL, Michel RP, Wang NS. Lingular lung biopsy - is it representative..JPG. Published online November 1985. https://pubmed.ncbi.nlm.nih.gov/4062039/

32. Cypel M, Liu M, Rubacha M, et al. Functional Repair of Human Donor Lungs by IL-10 Gene Therapy. *Sci Transl Med*. 2009;1(4). doi:10.1126/scitranslmed.3000266

33. Lin H, Chen M, Tian F, et al. α1-Anti-trypsin improves function of porcine donor lungs during ex-vivo lung perfusion. *J Heart Lung Transplant*. 2018;37(5):656-666. doi:10.1016/j.healun.2017.09.019

34. Iskender I, Sakamoto J, Nakajima D, et al. Human α1-antitrypsin improves early post-transplant lung function: Pre-clinical studies in a pig lung transplant model. *J Heart Lung Transplant*. 2016;35(7):913-921. doi:10.1016/j.healun.2016.03.006

Tables

Table 1. Donor lung characteristics for the mRNA study cohort.

Case #	Age	Sex	Donor Type	Cause of Death	Smoking History	Reason for Rejection	Last pO_2/FiO2 (mmHg)
1	29	M	DCD	Subdural hematoma	No	Contusion	360
2	40	M	DCD	Cardiac arrest	No	Logistical	402
3	69	F	DCD	Cardiac arrest	No	Emboli	475
4	23	M	DBD	Trauma	No	Edema	397
5	64	M	DBD	CVA	Yes	Smoking history	224
6	58	M	DBD	CVA	No	Infection	174
7	60	M	DBD	CVA	Yes	Emphysema	387
8	52	M	DBD	Trauma	Unknown	Lobar transplant	430

DCD = Donor after cardiovascular determination of death, DBD = Donor after brain death determination of death; CVA = cerebrovascular accident

Table 2. Global intra-lung CV (coefficient of variance) comparisons in different biopsy combinations by graft rejection type.

Biopsy Combination	Declined for Non-Graft Related Reasons (%CV [IQR])	Declined for Graft-Related Reasons (%CV [IQR])
S3 + S6 + S9	20.3 [13.1 – 35.2]	47.4 [33.2 – 68.4]
p value vs. intra-biopsy	0.80	<0.0001
S3 + S6 + S9 + Injury	50.6 [30.7 – 60.9]	57.3 [36.2 – 70.7]
p value vs. S3+S6+S9	0.019	0.52
S3 + S6 + S9 + Lingula	55.0 [23.2 – 78.4]	50.7 [38.0 – 67.1]
p value vs. S3+S6+S9	0.026	0.54

Table 3. *Donor lung characteristics for the EVLP protein study cohort.*

Case #	Age	Sex	Donor Type	Cause of Death	Smoking History	Indication for EVLP	Last pO_2/FiO_2 (mmHg)	EVLP Outcome
9	74	M	DCD	CVA	-	Adhesions, infiltrates	442	BLT
10	44	M	DBD	Anoxia	Active	Infiltrates	360	BLT
11	54	M	DCD	Anoxia	-	Endobronchial mucous	357	SLT (L)
12	54	M	DCD	MAID	Former	Endobronchial mucous	501	SLT (L)
13	28	M	DCD	Anoxia	Active	Suspected aspiration, infection	323	SLT (R)
14	23	M	DBD	Anoxia	-	Suspected aspiration, infection	463	Declined
15	40	M	DBD	CVA	Former	Pulmonary emboli	473	BLT
16	49	M	DBD	Trauma	Active	Suspected infection	379	BLT

BLT = bilateral lung transplantation, SLT (L) = single lung transplantation (left), SLT (R) = single lung transplantation (right), DCD = Donor after cardiovascular determination of death, DBD = Donor after brain death determination of death.

Figure Legends

Figure 1. Overview of the study including the central question, methods, results, and implications.

Figure 2. *Sampling Schematic.* (A) Representative image of whole lung slices #3, 6, 9, and the corresponding 1 cm^3 samples of tissues biopsied. Red circles illustrate the locations of tissue biopsies taken from donor lungs. (B) Donor lungs on the EVLP circuit, from which perfusates were extracted from each lobe venous drainage (RUL, RLL, LUL, and LLL).

Figure 3. *Cytokine levels in tissue biopsies.* Colour swarm plots for IL-6 (A), IL-8 (B), IL-10 (C), and IL-1β (D) mRNA measurements. Datapoints are color-coded by their sampling location in each donor lung (x-axis). The triplicates are sample replicates in each biopsy. The black solid lines show means within cases while the green dotted lines show the global mean across all cases.

Figure 4. *Cytokine levels in EVLP perfusate collected from different lung lobes.* Swarm plots for IL-6 (A), IL-8 (B), IL-10 (C), and IL-1β (D) protein measurements in EVLP perfusate fluid. Datapoints are color-coded by their sampling locations across individual donor lungs (x-axis). Technical replicates were performed in triplicate. The black solid lines show means within cases while the green dotted lines show the global mean across all cases.

Figure S1. *Cytokine gene expression levels in biopsy specimens.* The Tukey-Kramer pairwise comparison staircase diagrams for all biopsies and cytokines in Cases 1-8. Darker colours represent lower p values (threshold=0.05, 0.01, 0.001), red grids indicate cytokine levels were higher in the given row, and green grids indicate cytokine levels were higher in the given column. For the intra-biopsy equal-variance tests and ANOVA, the degree of freedom for the numerator of

528 the F distribution (i.e., for the between-group variation) is 4, and the degree of freedom for the
529 denominator of the F distribution (i.e., for the within-group variation) is 10.

530 Figure S2. *Normalized cytokine expression levels by biopsy locations.* Datapoints in the box plots
531 are normalized to the mean and standard deviation of the slices #3, 6, and 9 biopsies of the
532 corresponding donor and cytokine. All (A), IL-6 (B), IL-8 (C), IL-10 (D), IL-1β (E). *$p<0.05$,
533 **$p<0.01$, ***$p<0.001$