Development of In Vivo Lung Perfusion Strategy for the Treatment of Lung Metastases

by

Pedro Augusto Reck dos Santos

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Institute of Medical Science
University of Toronto

© Copyright by Pedro Augusto Reck dos Santos 2015
Development of In Vivo Lung Perfusion Strategy for the Treatment of Lung Metastases

Pedro Augusto Reck dos Santos
Masters of Science
Institute of Medical Science
University of Toronto
2015

Abstract: In Vivo Lung Perfusion (IVLP) can potentially enhance the treatment of lung metastases by allowing the delivery of elevated doses of chemotherapy to the lungs without systemic exposure. Previously, experimental and clinical studies reported questionable efficacy and frequent lung toxicity, hindering the broad application of this technique. We have developed a modified IVLP strategy that avoids acute lung injury using a protective mode of ventilation/perfusion. Our IVLP strategy enabled an extended perfusion time using chemotherapy doses usually given intravenously to patients. Additionally, we successfully administered higher chemotherapy levels in a safe manner and found higher lung tissue levels of chemotherapeutic drugs compared to previous studies. Importantly, these findings were possible without acute lung injury, demonstrating a likely protective effect of our IVLP strategy. This is a significant step towards a safer and more effective IVLP technique to help patients with pulmonary metastases, minimizing the adverse effects of chemotherapy to the lungs.
Acknowledgements

First, I would like to thank Dr. Marcelo Cypel, for giving me the opportunity to work in an exciting and innovative project. I think it is a very challenging task to develop an animal model and move the research forward and I am happy that Marcelo believed me capable of performing these important tasks. He always found time to discuss the successes and challenges that were faced during these years in the lab. His guidance and enthusiastic support helped me a great deal. We have been friends since we met in 2004 at the Hospital Sao Lucas - PUCRS and I am very happy to see his remarkable progress as a scientist. Without Marcelo’s help, my journey to Canada would not be possible and I would like to thank him especially for this opportunity.

I had the pleasure to work in the same lab as Dr. Shaf Keshavjee and Dr. Tom Waddell. With Dr. Keshavjee and Dr. Waddell, I learned how to efficiently guide a research project, how to see the “big picture” in a project and to pose the proper questions during research. I also learned about the current state of research, which involves collaboration with other groups as a key part for success. These successful surgeon scientists became my role model of a contemporary surgeon.

I must thank Dr. Marc de Perrot, Dr. Mingyao Liu, Dr. David Hwang and Dr. Natasha Leighl who gave invaluable insights on my research project. In addition, I had the privilege to meet, discuss and learn with surgeons Dr. Paul Van Schil and Dr. Thorsten Krueger, both experienced on clinical and experimental IVLP.
The collaboration between fellows is definitely one of the strengths of the Latner thoracic surgery labs. In this setting, I had the opportunity to work with colleagues (and friends) from many parts of the world including Japan, China, Turkey, Canada, Chile and, of course, Brazil. These talented people helped me throughout my research and I am sure that this project would not be possible without their help. It is my pleasure, to acknowledge and ensure that Jin Sakamoto, Lihua Song, Manyin Chen, Ilker Iskender, Daisuke Nakajima, Virginia Linaere, Kathy Chan, Chantel Arce, Tiago Machuca, Paul Chartrand, and Ivone Ornelas know how important they were for me during these years in the lab. I would also like to thank Golnaz Karoubi for editing of this thesis. Also it was a great pleasure to work with Barbara Bojko and German Augusto Gomez-Rios from the University of Waterloo during the experiments.

The resources were an important factor for this research project and I would like to thank XVivo Perfusion for their strong support. Also, I would like to thank the Princess Margaret Hospital (Innovation Grant) and especially the American Association for Thoracic Surgery (Michael E. DeBakey Research Scholarship).

One to the most difficult tasks for me was the management of the pigs and to help me in this major part of research, I had the support of a strong team: David Hanwell, Alyssa Goldstein, Shawna Vandenburg, Sandra Lafrance, Walter Ingles, Tihomir Dryanovski (Tiho) and Trista Murphy. I very much appreciate your help!

I had the privilege to be trained in general surgery at the Décima Enfermaria – Santa Casa de Porto Alegre and I would like to thank my mentors Dr. Roberto Pelegrini Coral (my first model of a surgeon-scientist), Dr. Fernando Krebs Cirne Lima and the rest of
the team. I am also very fortunate to have learned Thoracic Surgery with Dr. Jose Antonio Figueiredo Pinto, Dr. Jayme Rios, and Dr. Jayme Heck. I would like to thank them for helping me during residency and supporting my ideas to look for further training abroad.

A special tribute must be given to the use of animals in a research project. I recognize that animals are important for research and certainly they are one of the main reasons why science keeps moving forward, and helping people. But we must remember that the lives of these animals couldn’t be spared. I think that all efforts should be done in order to diminish the number of animals used for research projects and if this is not possible, we need to do our best so that no experiment is “wasted”. We must remember that one experiment is a life of one animal! I truly believe, and this is something that I will bring always with me, that all the experiments in which animals are used must to be done as if it was “the first case of your study”, with proper planning, commitment to surgical perfection, and a strong focus on the ethical and respectful management of the animals. I will always remember a Japanese fellow who praying the few instants before he euthanized a pig during an experiment – that was a great demonstration of respect and I always followed that attitude. So, I would like to give a great thanks to the pigs that were used in these experiments – they can be sure that I did my best and I recognize that they gave the greatest contribution to this project.
Dedication

This thesis is dedicated to my family: my parents Jorge and Maria Isabel, my sisters Mariana and Sofia and my grandfather Pedro, who gave me support and encouragement to live abroad, and to my fiancée Paula Baierle Losekann, who shared with me the successes and challenges with love, patience and support. Paula, I love you so much and I am sure that nothing would be possible without your support. I can’t forget our little dog Hanna, who came from Brazil to help us to adapt to a different country, bringing energy and happiness to our Canadian home. I also know that all my other dogs (Xuxa, Lita, Tila, Bela, Dora, Pretinha, Batalha) that are in Brazil are always with me.

“Whatever your dream is, you must dedicate to it a lot of your time and thoughts. You must have love and passion for what you are doing to find the energy, strength and dedication necessary to succeed in any type of activity. If you decide to fight for something, you have to work hard to win”

Ayrton Senna
# Table of Contents

1 **Introduction** ....................................................................................................................... 1
   1.1 **Overview of Lung Metastases** ......................................................................................... 2
       1.1.1 Metastatic dissemination ............................................................................................... 2
       1.1.2 The lung as a site for metastases ..................................................................................... 4
       1.1.3 Surgical treatment of lung metastases ............................................................................. 8
       1.1.4 Current limitations of lung metastasectomy ................................................................ 18
   1.2 **In Vivo Lung Perfusion for the Treatment of Lung Metastases** ..................................... 21
       1.2.1 A rationale for the use of high-dose chemotherapy to an organ .................................... 21
       1.2.2 Isolated Organ Perfusions – limbs and liver ................................................................ 23
       1.2.3 IVLP – Experimental and clinical background .............................................................. 24
           1.2.3.1 Experimental Studies ............................................................................................... 25
           1.2.3.2 Clinical studies ........................................................................................................ 35
       1.2.4 IVLP - A critic of studies performed to date ................................................................. 42
       1.2.5 The Ex Vivo Lung Perfusion strategy – a platform to develop an optimized IVLP strategy ................................................................................................................................. 46

2 **Rationale, Hypothesis, Objectives and Study Design** ......................................................... 49
   2.1 **Rationale** .......................................................................................................................... 50
   2.2 **Hypothesis** ........................................................................................................................ 50
   2.3 **Objectives** .......................................................................................................................... 50
   2.4 **Study Design** ...................................................................................................................... 51
       2.4.1 Phase I – Aims .................................................................................................................. 51
       2.4.2 Phase II – Aims ................................................................................................................ 52
       2.4.3 Phase III – Aims .............................................................................................................. 54

3 **Methods** .............................................................................................................................. 55
   3.1 **Animals** ............................................................................................................................. 56
   3.2 **Human lungs** ...................................................................................................................... 56
   3.3 **Experimental Strategy** ...................................................................................................... 56
   3.4 **IVLP Procedure** ............................................................................................................... 58
   3.5 **IVLP circuit** ....................................................................................................................... 61
   3.6 **Priming the circuit** ............................................................................................................. 63
   3.7 **Perfusion Strategy** ............................................................................................................ 63
   3.8 **Ventilation strategy for measurement of left lung physiology** ........................................ 65
   3.9 **Collection of data** .............................................................................................................. 66
   3.10 **Terminating IVLP and reperfusion phase** ...................................................................... 68
   3.11 **Lung function assessments** ............................................................................................ 68
   3.12 **Histology** ......................................................................................................................... 69
   3.13 **Radiologic Assessment** ................................................................................................... 70
   3.14 **Administration of chemotherapy** .................................................................................... 70
   3.15 **Pharmacokinetics of drugs** ............................................................................................ 71
   3.16 **Doxorubicin analysis in samples** .................................................................................... 72
       3.16.1 Sample processing: ......................................................................................................... 74
   3.17 **Ifosfamide analysis in samples** ....................................................................................... 76
       3.17.1 Sample processing: ......................................................................................................... 78
   3.18 **Tissue preparation for ELISA** .......................................................................................... 80
   3.19 **Tunel staining** .................................................................................................................. 81
   3.20 **Fluorescence** ................................................................................................................... 81
3.21 Statistical analysis ................................................................. 82

4 Results: ......................................................................................... 84
  4.1 Phase I - Development of the large animal model ......................... 85
  4.2 Phase II - Studies with chemotherapy ....................................... 90
  4.3 Phase III - Human lungs ......................................................... 111

5 Discussion: .................................................................................. 115

6 Conclusion .................................................................................. 132

7 Future directions .......................................................................... 135

8 Dissemination of Work .................................................................. 138
  8.1.1 Presentation in Meetings: ..................................................... 139
  8.1.2 Publications: ....................................................................... 139

9 Appendices ..................................................................................... 140
  9.1 Doxorubicin (Adriamycin) ....................................................... 141
  9.2 Ifosfamide ................................................................................ 142

10 References .................................................................................... 143
# List of Tables

<table>
<thead>
<tr>
<th>Number</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Criteria for lung metastasectomy</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>Clinical IVLP studies performed</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>Ventilatory strategy used for IVLP</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>Composition of Steen solution</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>Perfusion strategy developed for the IVLP technique</td>
<td>64</td>
</tr>
<tr>
<td>6</td>
<td>Comparison of the previous large animal IVLP studies with Doxorubicin</td>
<td>126</td>
</tr>
<tr>
<td>Number</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>The wide spectrum of disease in relation to lung metastases.</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>The importance of a complete resection in the management of lung metastases</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>The importance of the number of lung metastases resected in relation to the prognosis</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>Extended disease free interval contributes to an improved survival in the management of lung metastases</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>Histologic types of lung metastases and the implications in the prognosis</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>Model of left lung IVLP in rats</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>IVLP technique used in clinical setting.</td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>Lung injury reported in IVLP.</td>
<td>43</td>
</tr>
<tr>
<td>9</td>
<td>The Toronto EVLP strategy.</td>
<td>47</td>
</tr>
<tr>
<td>10</td>
<td>Left atrium exposed, with upper and lower veins identified and encircled</td>
<td>59</td>
</tr>
<tr>
<td>11</td>
<td>Isolation of bronchial circulation</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>IVLP strategy developed</td>
<td>62</td>
</tr>
<tr>
<td>13</td>
<td>Collection of data for IVLP</td>
<td>67</td>
</tr>
<tr>
<td>14</td>
<td>Left lung function during IVLP</td>
<td>85</td>
</tr>
<tr>
<td>15</td>
<td>Left lung function during the entire procedure</td>
<td>86</td>
</tr>
<tr>
<td>16</td>
<td>Histologic assessment of the perfused lung</td>
<td>87</td>
</tr>
<tr>
<td>17</td>
<td>Chest X-ray of an animal subjected to 4 hours of IVLP and 4 hours of reperfusion</td>
<td>88</td>
</tr>
<tr>
<td>18</td>
<td>The distribution of India Ink after IVLP</td>
<td>89</td>
</tr>
<tr>
<td>19</td>
<td>Left lung function during IVLP with Doxorubicin 75 mg/m²</td>
<td>91</td>
</tr>
<tr>
<td>20</td>
<td>Airways dynamics during IVLP with Doxorubicin 75 mg/m²</td>
<td>92</td>
</tr>
<tr>
<td>21</td>
<td>Histologic assessment of the perfused lung with Doxorubicin 75mg/m²</td>
<td>93</td>
</tr>
<tr>
<td>22</td>
<td>Chest X-ray of an animal subjected to 4 hours of IVLP and 4 hours of reperfusion</td>
<td>94</td>
</tr>
<tr>
<td>23</td>
<td>Left lung function during IVLP with two drugs</td>
<td>95</td>
</tr>
<tr>
<td>24</td>
<td>Airway dynamics during IVLP with two drugs</td>
<td>96</td>
</tr>
<tr>
<td>25</td>
<td>The histologic assessment of the lung perfused with two drugs</td>
<td>97</td>
</tr>
<tr>
<td>26</td>
<td>Lung function of the two animals submitted to IVLP with Doxorubicin 150 mg/m²</td>
<td>98</td>
</tr>
<tr>
<td>27</td>
<td>Histologic and macroscopic assessment of the lung perfused with Doxorubicin 150 mg/m²</td>
<td>99</td>
</tr>
<tr>
<td>28</td>
<td>Lung function of the two animals submitted to IVLP with Doxorubicin 225 mg/m²</td>
<td>100</td>
</tr>
<tr>
<td>29</td>
<td>Macroscopic signs of lung injury verified during IVLP with Doxorubicin 225 mg/m²</td>
<td>101</td>
</tr>
<tr>
<td>30</td>
<td>Inflammatory profile in lung tissue</td>
<td>102</td>
</tr>
<tr>
<td>31</td>
<td>Apoptosis Imaging</td>
<td>103</td>
</tr>
<tr>
<td>32</td>
<td>Tissue and perfusate levels measured in experiments with Doxorubicin 75 mg/m²</td>
<td>104</td>
</tr>
<tr>
<td>33</td>
<td>Pharmacokinetics of Ifosfamide</td>
<td>105</td>
</tr>
<tr>
<td>34</td>
<td>Comparison in the perfusate and tissue levels of Doxorubicin obtained in the dose-escalating IVLP experiments</td>
<td>106</td>
</tr>
<tr>
<td>35</td>
<td>Influence of duration of IVLP in the final concentration of Doxorubicin in lung tissue</td>
<td>107</td>
</tr>
<tr>
<td>36</td>
<td>Auto fluorescence of Doxorubicin</td>
<td>108</td>
</tr>
<tr>
<td>37</td>
<td>Influence of bronchial circulation in the final tissue levels of Doxorubicin</td>
<td>110</td>
</tr>
<tr>
<td>38</td>
<td>Pharmacokinetics of chemotherapy in a lung-free circuit</td>
<td>111</td>
</tr>
<tr>
<td>39</td>
<td>Human lungs perfused Ex Vivo with chemotherapy</td>
<td>112</td>
</tr>
<tr>
<td>40</td>
<td>Function of the human lungs submitted to EVLP with Doxorubicin</td>
<td>113</td>
</tr>
</tbody>
</table>
## List of Appendices

<table>
<thead>
<tr>
<th>Number</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Doxorubicin</td>
<td>141</td>
</tr>
<tr>
<td>2</td>
<td>Ifosfamide</td>
<td>142</td>
</tr>
</tbody>
</table>
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIM 75/9</td>
<td>Chemotherapeutic scheme of Adriamycin, Ifosfamide and Mesna</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Body surface area</td>
</tr>
<tr>
<td>CAPCR</td>
<td>Coordinated approval process for clinical research</td>
</tr>
<tr>
<td>Cdyn</td>
<td>Dynamic compliance</td>
</tr>
<tr>
<td>Cstat</td>
<td>Static compliance</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EVLP</td>
<td>Ex vivo lung perfusion</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>FOLFOX</td>
<td>Chemotherapeutic scheme of folinic acid, fluorouracil, and oxaliplatin</td>
</tr>
<tr>
<td>Fr</td>
<td>French (system commonly used to measure the size of a catheter)</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IL1-β</td>
<td>Interleukin one-beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin six</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin eight</td>
</tr>
<tr>
<td>IVLP</td>
<td>In vivo lung perfusion</td>
</tr>
<tr>
<td>LA</td>
<td>Left atrium</td>
</tr>
<tr>
<td>m²</td>
<td>Meters square of body surface</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimeters of mercury</td>
</tr>
<tr>
<td>PA</td>
<td>Pulmonary artery</td>
</tr>
<tr>
<td>Pawp</td>
<td>Peak airway pressure</td>
</tr>
<tr>
<td>P/F ratio</td>
<td>Partial pressure of oxygen divided by the fraction of inspired oxygen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>PCO₂</td>
<td>Partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>Pplat</td>
<td>Plateau airway pressure</td>
</tr>
<tr>
<td>PVR</td>
<td>Pulmonary vascular resistance</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase micro extraction</td>
</tr>
<tr>
<td>TNF alfa</td>
<td>Tumor necrosis factor - alfa</td>
</tr>
<tr>
<td>VATS</td>
<td>Video assisted thoracic surgery</td>
</tr>
<tr>
<td>µg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius degrees of temperature</td>
</tr>
</tbody>
</table>
1 Introduction
1.1 Overview of Lung Metastases

1.1.1 Metastatic dissemination

Cancer is the second leading cause of death in the world and the majority of causes of the cancer-related deaths are due to the dissemination of the tumor to distant areas in the body, a process called metastatic spread. The impact of this dissemination on the prognosis of patients is enormous, dramatically impairing the survival and the quality of life[1].

A succession of events is necessary for a tumor to metastasize. Although this complex process is not entirely understood, initially local invasion of surrounding tissues is necessary in order to initiate the process of dissemination. Then, intravasation of the tumor cells in the lymphatic or blood vessels must happen. Tumors typically spread through the lymphatic route (malignant tumors spread to regional lymph nodes, following the natural routes of lymphatic drainage of the primary organ affected) or the hematogenous route (cancer cells arise in the systemic circulation and dissemination may occur to organs like the liver and the lungs, which are common sites of spread because they receive most of the blood that comes from the portal system and caval circulation respectively). These two main routes can overlap, considering the numerous interconnections between the lymphatic and the vascular system[2].

Once these cells reach the circulation, they will arrest at a distant organ site and must be able to survive in a different environment[3]. The venous drainage of a specific tumor may play a role in this arrest, and examples of this are gastro-intestinal cancers, which
have their blood flow drained through the portal system, and as a consequence, these
tumors typically metastasize to the liver. In a sequence of events, after the arrest of these
circulating cancer cells in a specific niche, tumors must be able to extravagate from the
microvasculature of the target-organ, entering in a new environment, where survival will
become a critical step. Overcoming the survival challenge, tumor cells will multiply,
creating micro metastatic foci of disease. The endpoint of this complex process is the
emergence of macroscopic metastases[4]. The barriers to infiltrate and the composition of
the microenvironment of each organ are unique, which means that metastases to different
organs may require distinct steps.

The microenvironment of each organ is also an important issue in the metastatic
dissemination and may help to explain why specific types of tumors cells have a
tendency to metastasize only to organs where they find a proper environment,
irrespective of the vascularization of the potential metastatic site of spread and the
amount of cancer cells delivered to a target-organ. This was addressed by the “seed an
soil” hypothesis, which emphasizes that the process of metastases selects cells that can
potentially embolize and survive in the circulation and effectively replicate within
another organ (the seed) and also gives importance to the microenvironment of each
organ (the soil). The interactions between the cancer and the host cells that will occur in
this microenvironment will allow the development of metastatic disease in sites where
endothelial cells and local growth factors of the host can effectively interact with the
circulating cancer cells[5].
1.1.2 The lung as a site for metastases

The lungs, in order to oxygenate the blood from the body, receive their blood flow from the entire systemic circulation through the upper and lower vena cava, via the pulmonary artery (PA) system. As a consequence, the lung actually serves as a “filter”, constituting a site where malignant tumour cells from cancers located in different areas of the body seed and develop. As a result, the lungs are a frequently site for metastatic disease. A characteristic pattern of the metastatic lung tumours is that they are most commonly encountered in the lower lobes of the lungs and this is explained because the lower parts of the lungs are more vascularized in comparison to the upper parts, which typically present with a higher ventilation/perfusion rate[6].

Lung metastases from extra pulmonary malignancies can be found in 20 – 54% of patients with cancer [7,8] and the malignant tumors that most commonly spread to the lungs are breast, colon, kidney, uterine malignancies and head and neck cancers. These tumors are more commonly found in the lungs because the incidence of these tumors is very high in the population and frequently they metastasize to other sites of the body as well. There are, however, tumors that have a great tendency to metastasize to the lungs preferentially, like choriocarcinomas, sarcomas, testicular tumors[9]. These tumors are not so commonly found in the population, but their strong tendency to spread almost exclusively to the lungs is remarkable.

Lung metastases can have a wide spectrum of presentation, ranging from a massive dissemination to even isolated lung metastases (Figure 1). These differences interfere in the prognosis and in the management of the patient.
Figure 1 - The wide spectrum of disease in relation to lung metastases. (A) Chest CT of lungs with multiple lung metastases (B) Chest CT of lungs with a single lesion, highlighted in red.

Considering the lung as the reference, an interesting pattern of dissemination of cancers was described according to the type and location of the primary tumor[10]:

1. Tumors that generally metastasize first to the lungs, then to other organs: sarcomas, melanomas, choriocarcinomas, thyroid, adrenal glad tumors, germ cell tumors and head and neck cancers.

2. Tumors that metastasize to the lungs at a more advanced stage, for instance after spreading to the liver: colon, stomach and pancreas. These tumors have their blood flow drained by the portal venous system, so typically the dissemination happens first to the liver, then to the lungs.

3. Tumors that simultaneously seed multiple organs: bladder, kidney, uterus and cervix. These tumors metastasize less frequently to the lungs in comparison to
the first two groups and dissemination happens through the blood and lymphatic systems.

4. Tumors that infrequently metastasize to the lungs: prostate, which has a preference to disseminate to the bones.

Breast cancer is considered unique because it is one of the most common extra thoracic tumors to spread to the lungs, but has a more complex pattern of spread.

In summary, the lungs are commonly affected by metastatic dissemination of malignant tumors and the concomitant association of spread to other organs like liver, brain and bones is common, which makes this clinical problem particularly challenging to treat. Isolated spread to the lungs does not occur frequently, but some tumours may have this characteristic and in this case, a select few patients may benefit from the resection of these isolated foci of disease.

The nutrition of the lung metastases and primary lung tumours is an interesting aspect, because of the dual blood circulation of the lungs. Besides PA system, which is responsible for the oxygenation of the blood of the entire body, there is the bronchial artery system, whose main role is to nourish the bronchial system with oxygenated blood. In a normal situation, the bronchial circulation receives approximately 1-2% of the total cardiac output (CO).[11]

This dual blood circulation of the lung raises questions about the nutrition of tumours in the lungs. Studies done in lungs of patients who succumbed to primary lung tumours verified that the major blood supply that nourished these tumours was from the bronchial
circulation[12]. Selective angiography done by the bronchial arteries of patients with primary lung cancers [13] confirmed this, but the pulmonary circulation was responsible for a small percentage of the vasculature as well.

On the other side, lung metastases receive their major blood supply from the PA circulation[14,15,16]. Primary lung tumours had a dominant bronchial circulation pattern and communications with the PA circulation are common, while pulmonary metastases had a pattern related to PA circulation, and communications were almost not seen[17]. Primary lung tumours may present a bronchial / mixed pattern of vascularization whereas lung metastases may have a predominant pulmonary vascularization. An interesting study was done using dynamic CT to analyse the vascularization of tumours of the lung. While an aortic pattern of vascularization was found in 72% of patients with primary lung cancer, the metastatic lung tumours only presented this pattern in 16% and 75% of PA perfusion pattern[18].

In experimental models of induced-lung metastases, where the pattern of vascularization was assessed, exclusive pulmonary circulation was found in 48%, mixed pulmonary and bronchial circulation in 36% and exclusive bronchial circulation in 16% only. Interestingly, metastases close to the hilum of the lung (in more central areas do the lung) received their blood supply predominantly from the bronchial circulation whereas peripheral metastases were nourished by the pulmonary circulation mainly[19]. In another study, after induction of lung metastases by intravenous injection of tumour cells, the ligation of the left PA diminished the burden of metastatic disease in this lung compared to controls[20], which suggests that lung metastases were dependent on
pulmonary blood flow to grow and corroborates the finding that lung metastases that grew after tumour cells were injected intravenously were associated with nourishment from the PA system[21].

These facts support the concept of delivering of chemotherapeutic drugs through the PA to treat lung metastases, as opposed to bronchial circulation perfusions, which are usually the route used for studies that target primary lung tumours. Bronchial circulation may contribute to the nutrition of lung metastases, but it is much more frequently associated with the vascularization of primary lung cancers and in a clinical setting, the administration of drugs through the bronchial arteries is a strategy focused on the treatment of primary lung cancer. [2,22-24]

1.1.3 Surgical treatment of lung metastases

Lung metastases are considered a systemic disease and usually treated with chemotherapy. However, a wide spectrum of response to the chemotherapeutic drugs in the management of lung metastases is observed, ranging from tumors where the efficacy of chemotherapy is questionable, like sarcomas[25] to cancer where chemotherapy is of paramount importance, like germ cell tumors.[26]

The surgical resection of lung metastases is a therapy accepted for the management of this disease. However, considering that these patients commonly present with dissemination to other organs as well, selection criteria were established in order to better select patients who will benefit from this invasive approach[27].
Criteria for indication of lung metastasectomy

<table>
<thead>
<tr>
<th>The primary tumor is controlled or can be controlled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete resection of the metastatic disease is possible</td>
</tr>
<tr>
<td>No extra thoracic disease is present <em>(liver metastases in colorectal cancer are an exception)</em></td>
</tr>
<tr>
<td>Clinical status of the patient</td>
</tr>
</tbody>
</table>

Table 1 – Criteria for lung metastasectomy [28].

The evaluation of prognostic factors among the population of patients who were submitted to lung metastasectomy is also important, because it allows the identification of patients who may actually benefit from this approach. A large series of 5206 patients submitted to lung metastasectomy was published [28], in order to assess the results of this approach. The findings from this publication allowed the identification of factors that were associated with better outcomes, such as:

*Complete resection:* the importance to resect completely the metastatic disease was described as a strong prognostic factor associated with improvement in survival. It is one of the key principles of lung metastasectomy and is highlighted in Figure 2.
Figure 2 – The importance of a complete resection in the management of lung metastases (from Pastorino[28]).

Complete resection of the metastatic disease remains as one of the most important principles in the surgical management of lung metastases. However, it is hard to estimate the proper number of lung metastases preoperatively, due to the small dimension of nodules that can be found during surgery. This fact may lead to incomplete resections due to the complexity of lung metastases presentation[29].

Number of nodules: Isolated lung metastases have a better prognosis than multiple tumors and the overall survival decreases proportionally to the number of lesions. There is no strict number of nodules that may contraindicate surgery, but the smaller the number of lesions to be resected, the better the prognosis (Figure 3).
Figure 3 – The importance of the number of lung metastases resected in relation to the prognosis. 95% Confidence Interval estimated by the Cox proportional hazard model was 0.681-0.818 for single lesions. Relative Risk = 0.7 for single versus multiple lung metastases (from Pastorino[28]).

*Disease free interval*: the time between the treatment of the primary tumor and the emergence of metastatic disease is called the disease-free interval (DFI). In respect to the 5-year follow-up, lung metastases with an extended DFI clearly had an increased survival in comparison to tumors where the lung metastases presented at shorter DFI and synchronic presentation, which usually are indications of a more aggressive biology of disease as highlighted on Figure 4. A DFI interval > 36 months clearly associates with a less aggressive pattern of dissemination when compared to a clinical scenario where the tumor recurs immediately or shortly after the resection of the primary disease.
Extended DFI contributes to an improved survival in the management of lung metastases. 95% Confidence Interval estimated by the Cox proportional hazard model was 0.57-0.7 for DFI > 36 months. Relative Risk = 0.63 for DFI > 36 versus DFI < 36 months (from Pastorino[28]).

Histology of the tumor: in patients submitted to resection of lung metastases, sarcomas and colorectal carcinomas are the two histological types of cancers more commonly found and unfortunately they are associated with a very poor survival rate and are typically resistant to chemotherapy. Melanomas also carry a poor prognosis due to the aggressive nature of this tumor as well as the same aforementioned resistance to chemotherapeutic drugs. On the other side, germ cell tumors can be considered an exception, because they show a very good response to systemic chemotherapy. Different types of cancers can definitely influence the prognosis of patients and the figure 5 highlights this aspect.
In summary, these prognostic criteria help to select potential candidates who may benefit from lung metastasectomy. As an example, a patient with a single lung metastases, extended DFI and favorable histology will have a better outcome from lung metastasectomy compared to a patient with a large number of nodules, short DFI and tumors such as sarcomas or melanomas.

Most of the experience in lung metastasectomy is based on series of cases, retrospective studies and a small number of prospective studies. In general, considering the lack of efficacy that current regimens of chemotherapy have in the management of lung metastases, being more palliative than curative, a presence of a small number of lung
metastases, a long disease free interval and the ability to perform complete resection of
the metastatic burden favors the indication for resection of the disease. However, there
are no strict criteria to include or exclude potential candidates for lung metastasectomy.

Regarding the tumors most commonly found in a large series of 5206 patients submitted
to lung metastasectomy, sarcomas and epithelial cancers (mainly colorectal carcinomas)
are the main types. Germ cell tumors, melanomas, kidney tumors, head and neck cancer
were other tumors treated, but at lower rates[28]. The same pattern of was found in
another large study, where the analysis of 575 patients described sarcomas and colorectal
carcinomas as the most common types among patients submitted to surgical resection of
lung metastases, with breast, urothelial, gynecological, head and neck, melanoma and
germ cell tumors described at lower incidences. [30]

The current results of lung metastasectomy according to the tumors more commonly
treated with this approach are discussed below:

*Soft tissue sarcomas:* rates of 46% of survival after lung metastasectomy at 3 years were
possible when resection of complete disease was done and a DFI larger than 12 months
was found[31]. In another study that reported a 5-year survival of 50.1%, besides the
DFI, single sided metastases, negative margins at resection and multiple lung
metastasectomies were associated with better survival[32]. In certain cases, even the
pediatric population of patients suffering from sarcomas benefit from lung
metastasectomy[33]. Sarcomas in general do not respond well to chemotherapy[34] and
lung metastasectomy remains as the only “curative” alternative for these patients.
Colorectal: Aside from soft tissue sarcomas, colorectal carcinoma is one of the most common indications for lung metastasectomy. Satisfactory 5-year survival, with rates of 40% could be found especially for patients where complete resection was possible[35]. A study by Blackmon et al. analyzed the prognostic factors for recurrence after lung metastasectomy and identified patients over 60 years of age, male sex and greater than 3 lesions as negative prognostic factors. The number of lesions and a preoperative DFI less than 3 years predicted lung recurrence after the first metastasectomy [36]. The size of the tumor, levels of carcinoembryonic antigen before the thoracotomy and the presence of metastatic disease are other factors that are associated with a poor prognosis.

Germ Cell: Surgical resection for metastatic testicular nonseminomatous germ cell tumors (NSGCT) offers an excellent prognosis, with a 5-year survival of 68%. Most tumors resected were teratomas (52.7%) and necrosis (21.5%) in this series with the pathology of the residual disease being an important factor, with persistent NSGCT and degenerative non-germ cell cancer correlating with worst outcome[37]. Nevertheless these unfavorable histologies may benefit from surgical resection, reaching a 5-year survival of 42.3% with the surgical resection. However, older age, pulmonary (compared to mediastinal disease) and more than 4 lesions were factors associated with shorter survival rates[38].

Osteosarcoma: A 5-year survival of 37% was found in this population of patients with metastatic disease restricted to the lungs. A DFI interval longer than 24 months positively influenced the prognosis and the presence of synchronic resectable lung metastases had a negative effect on survival. Resection of local recurrences after the first
lung metastasectomy did not impact the survival and should be indicated, whenever feasible[39]. The number of lung metastases and the ability to perform a complete resection were important prognostic factors in this type of tumor as well[40].

*Breast Cancer:* Patients with isolated lung metastases are a very uncommon clinical situation, but these patients may benefit from lung metastasectomy, achieving a mean 5-year survival of 54.5%, and the prognostic factors identified have been a DFI smaller than 36 months and the ability to complete the pulmonary resection[41]. Lung metastasectomy improved survival compared to no metastasectomy in a matched analysis regarding the age and the staging of the breast cancer. Also, an increase in the overall survival in the group submitted to lung metastasectomy was identified in those patients with complete resection of the metastatic burden, isolated, size of the lung metastases (>3 cm) and hormone positive receptor status[42,43].

*Melanoma:* Due to the high tendency of early dissemination of melanoma, the indication for lung metastasectomy is controversial. Very select patients, with isolated metastases with a size smaller than 2 cm, where complete resection with a negative margin is possible, may benefit from this approach. It has been reported that these patients have a 5-year survival rate of 38% after complete lung metastasectomy [44,45]. Patients with good performance status, with a limited number of lung metastases and a long DFI may achieve a 4-year survival rate of 23%[46]. Isolated lung metastases and the completeness of resection were identified as very important prognostic factors.[47]

*Gynecologic malignancies:* In a study where 70 patients with metastatic disease restricted to the lungs submitted to resection, a 5-year survival of 46.8% was found. Survival was
negatively influenced by a disease-free interval smaller than 24 months and a primary tumor with primary origin in the uterine cervix area[48]. In this setting, the disease-free interval showed to be an important factor associated with survival in another publication, where a disease-free interval smaller than 12 months impaired the survival[49].

_Gastric cancer:_ Resectable lung metastases from gastric cancer are an uncommon finding, but in selected cases, especially when the disease-free interval is longer than 12 months found a reasonable 5 year survival[27]. In another report, small and isolated lung lesions, with an extended disease-free interval demonstrated an overall survival of 55%[50].

_Head and Neck:_ lung metastasectomy impacted survival when compared to best clinical supportive care and in the patients submitted to lung metastasectomy, a disease-free interval longer than 21.5 months benefited most[51]. A 5-year survival of 26.5% was described on another study, and negative prognostic factors were identified were male sex, oral cavity cancers, incomplete resection of lung metastases and lymph node metastases[52].

_Kidney carcinoma:_ resection of lung metastases from renal cancer can achieve a 5-year survival rate of 49%. Age, female gender and more than 2 lesions were factors associated with a decrease in survival[53]. In another study, the presence of lymph nodes metastases, a disease-free interval longer than 24 months and the number of metastases influenced the prognostic of patients with resectable pulmonary disease, and metastasectomy showed to be a safe an effective way to treat this problem[54-58]. New targeted therapies may influence the future approach for this disease[36,59-62].
In summary, many types of tumors can be treated with lung metastasectomy, benefiting patients when the proper selection criteria are followed.

The resection of the lung metastases can usually be performed with wedge resections, which allows complete resection of disease and preservation of lung parenchyma, whereas more complex resections like lobectomy or pneumonectomy may be indicated according to the size and location of the lesions[42,63,64].

1.1.4 Current limitations of lung metastasectomy

Despite improvements in detection methods and chemotherapeutic regimens available for neoadjuvant or adjuvant treatment, the overall survival of patients subjected to lung metastasectomy remains at best within a range of 30-40%. However, among the entire population of patients submitted to lung metastasectomy, a wide spectrum of responses to the treatment can be found.

Patients with “favorable” prognostic factors like those with single lung metastases, prolonged disease-free interval between primary tumor and metastatic dissemination, absence of thoracic node involvement[44,65] in general have better outcomes. In contrast, patients with three or more nodules and shorter disease-free intervals are less likely to benefit from lung metastasectomy[66,67], and considering that the benefit of chemotherapy after resection of lung metastases is unclear, the indication for treatment is sometimes controversial.
The criteria to separate patients who are candidates from those who can benefit less from surgery are not clear, and the decisions are based on factors like the number of lesions, the disease free interval, the type of tumors, the presence of metastatic regional lymph nodes. Patients who are “borderline” candidates, like those with many pulmonary nodules, or with short disease-free intervals to surgical treatment are a matter of discussion because the indications for lung metastasectomy are not clear in this setting. Of note, there are many clinical situations where the exclusion of patients as potential candidates for lung metastasectomy is not clear. The ability to complete resection, the number of nodules and short interval between the resection of the primary tumor and the emergence of metastatic disease are factors that may influence this decision currently chosen in a subjective way.

The evidence that supports the indication for lung metastasectomy is based on a large number of publications such as series of cases, retrospective and some prospective studies. There is no randomized trial that gives support to lung metastasectomy, leading to criticism about the efficacy of the procedures, especially for colorectal cancers[68].

Once lung metastasectomy is performed, the recurrence of the resected disease may occur, constituting one the main factors associated with poor survival. Unfortunately the use of chemotherapy as a neoadjuvant or adjuvant treatment associated with lung metastasectomy showed efficacy only in a few instances. Systemic chemotherapy is important in the multimodality management tumors like osteosarcomas[69,70], breast cancer[71,72], choriocarcinomas[73,74] and germ cell tumors [75,76], whereas for
tumors like soft tissue sarcomas[25,77] and colorectal carcinomas[78,79], the benefit in prolonging survival is still unclear.

Relapse at multiple distant sites is already a problem hard to treat considering the current chemotherapeutic regimens available and their low efficacy. Epithelial cancers and melanomas present with extra thoracic relapse after first lung metastasectomy in 56% and 73% of cases respectively, whereas in sarcomas, the intrathoracic relapse after lung metastasectomy represents the majority of cases[28,78].

While the dissemination to many sites is particularly challenging to treat, the intrathoracic recurrence could potentially be targeted. The cause of local recurrence is generally attributed to micrometastatic disease, which are probably in the lungs at the time of resection but cannot be noticed by palpation of the lung tissue neither during preoperative image exams. The inability to reach adequate levels of chemotherapy in lung tissue with conventional systemic chemotherapy administered after lung resection may also contribute to the emergence of local recurrence after lung metastasectomy.

Pulmonary relapse after the first lung metastasectomy is a problem very commonly found. For example, in sarcomas it was described to happen within a range of 41% to 83% [54,56-58,80] while patients with metastatic colorectal carcinomas, the local recurrence rates were 26-56%[36,59,61,62,81,82]. Of note, the colorectal cancer recurrence is more frequently associated to concomitant dissemination to other sites.

Once recurrence develops in the lung after the first metastasectomy, the resection of metastatic disease, whenever feasible, is indicated, but often patients became unresectable[28,63,64]. Thus, a strategy that can diminish the rates or even prevent the
local recurrence after lung metastasectomy will greatly help the patients who are in this situation.

1.2 In Vivo Lung Perfusion for the Treatment of Lung Metastases

1.2.1 A rationale for the use of high-dose chemotherapy to an organ

The principle to administer elevated doses of chemotherapy to a specified target-organ compromised by metastatic or locally advanced disease is based on the assumption that very high levels of chemotherapy directed to a specific target may be more effective than conventional systemic administration and without the hazardous systemic side effects of chemotherapeutic agents. In fact, the systemic side effects are often the limitation for the administration of higher doses of chemotherapy in a clinical setting. There is a need to balance the amount of drug administered with the effects that this drug may have in the body. In theory, the administration of chemotherapy exclusively to an organ, circulating the drug within a closed circuit, overcomes this problem because allows the target-organ to be exposed to high levels of the medication, and limits the systemic exposure, because circulates the drug in a closed system with no leak for the systemic circulation.

Using this principle, a primary cancer that spreads exclusively to a specific site, like the lungs or the liver, could achieve a very high rate of cure if local recurrence could be
prevented after the initial metastasectomy. Examples of patients who may benefit from this approach are those with like metastatic sarcomas to the lungs, metastatic colorectal cancer to the liver and metastatic disease from melanomas restricted to a limb.

An ideal approach could be resection of all macro metastatic disease and treatment of the micrometastatic disease with high-doses of chemotherapy administered to the specific site of disease (and without the side effects of the chemotherapeutic drugs). This approach could potentially diminish the chances of local recurrence that often happens after lung metastasectomy and impairs the prognosis of the patients who are suffering from lung metastases.

The concept to perfuse and organ in situ, administering high-doses of chemotherapy with minimal systemic exposure, is not new and was already tested in many sites like the limbs, liver, lungs. Specifically considering the lung, IVLP consists in the cannulation of a main pulmonary artery (inflow) that nourishes one lung and cannulation of the pulmonary veins that drain the blood from this lung (outflow). With the control of the inflow and the outflow of the right or the left lungs, we can exclude him from the systemic circulation and perfuse it within this closed system, which constitutes a platform for several local therapies, like chemotherapy in the case of lung metastases. Previously, IVLP showed to be an efficient way to achieve higher levels of chemotherapy in lung tissue, when compared to the conventional systemic administration[65,83], reaching up to 25 times more chemotherapy levels in normal lung tissue and less Doxorubicin levels in the heart compared to systemic administration of this drug[66,84].
This approach addresses one of the weaknesses of the conventional intravenous administration of chemotherapy, which is the ability to deliver very high (and presumably more effective) levels of chemotherapy in target organs. Once a drug is administered intravenously, only a small portion reaches the site of disease. [69]. Thus, the isolated organ perfusion represents an interesting concept that can potentially offer local therapy for cancer, achieving elevated levels of drug in lung tissue and a good distribution of the drug in the organ.

1.2.2 Isolated Organ Perfusions – limbs and liver

The perfusion of an organ within a closed circuit was described by Carrel and Lindbergh using a device developed to enable long-term organ culture, called the Lindbergh – RIMR pump[71]. With this device, from April 1935 to May 1939, 898 perfusion experiments were done and perfusion was carried out mainly in hearts of cats and kittens, with the perfusate being constitute of Tyrode’s solution with 50% of serum at 37 °C of temperature and surprisingly the hearts were able to maintain contraction for up to 12 hours[73].

Creech described many cases of isolated organs perfusions where the main indication was the perfusion of the limbs for advanced melanomas and soft tissue sarcomas[75] while perfusion of the lung was associated with elevated mortality rates[77]. Over time, the limbs and liver were more commonly explored in a clinical setting, in comparison to the lungs, and the toxicity reported in lungs is probably the main reason for this.
Isolated perfusion of the limbs can be achievable in the upper and lower extremities, due to easy access to the vessels of the extremities. The use of this strategy as a prophylactic measure in patients with high-risk melanomas of the extremities was assessed in large randomized trial, but no disease-free interval or increase in the overall survival was identified[85]. Therapeutic limb perfusion, indeed, is a strategy that allows sustained loco regional control for multiple in-transit melanomas metastases[86]. Additionally, it is currently used as an induction method for patients with locally advanced soft tissue sarcomas of the extremities, with a high salvage rate of the limbs.[87]

Isolated Liver Perfusion was also studied and focused on the management of malignancies like metastatic colorectal carcinomas[88,89], multiple advanced hepatocellular carcinoma (as an adjuvant measure to reductive hepatectomy)[90] and metastatic ocular melanoma with promising results. The greatest benefit was found in studies done with metastatic ocular melanoma, suggesting an increase in the survival of these population of patients in this setting[91].

1.2.3 IVLP – Experimental and clinical background

The lung was a potential target to be perfused in an isolated fashion, because the inflow (PA) and the outflow (pulmonary veins) could be surgically controlled, isolating the lung is situ, allowing the perfusion of the organ with different modalities of treatment. The IVLP was extensively studied in the past in an experimental setting and some clinical experience also exists.
1.2.3.1 Experimental Studies

Initial experimental studies were published by Pierpont in 1959[78], in dogs which lungs were perfused with nitrogen mustard with a dose of 0.4 mg/kg of total body weight and they used a recirculating system that aided a negative pressure in the outflow of blood from the pulmonary veins. Blood and heparin were used as the perfusate solution and a flow rate of 175 ml/min was chose to avoid lung edema. Perfusion time was 15 minutes and radioactive red blood cells were added to the perfusate in order to analyze systemic leak of the circuit. Oxygen was provided from the ventilator used and not from the perfusate. Histologic analysis showed acute pneumonitis with resolution, in a sequence of vascular effusion, necrosis, white blood cells infiltration and fibrosis. They found that there was not escape from the perfusion circuit to the systemic circulation and, despite the reported lung injury; administration of high dose chemotherapy was possible in this setting.

An important concept was introduced by Jacobs[78] in a large animal model which was the drainage by gravity from the pulmonary veins to the reservoir. With this strategy, even higher perfusion flow rates could be tolerable without the appearance of lung edema and a higher dose of nitrogen mustard could be administered (1.6mg/kg).

A technique which allowed the study of this approach in small animals [80,81,82] was very useful to investigate the pharmacokinetics of drugs using IVLP and especially, to analyze the efficacy of this therapy in vivo, using models of induced metastatic cancer to the lungs and analyzing the response to the therapy in vivo (Figure 6).
The efficacy of this therapy could be assessed in small animal models of induced-lung metastases. In this setting, sarcomas and colorectal carcinomas lung metastases were induced experimentally in rats and extensively studied. These two types of tumors are the most prevalent histologies found in patients submitted to lung metastasectomy[28], making them potential targets for future clinical studies.

Regarding sarcomatous lung metastases, IVLP demonstrated to be effective in eradication of the tumors using Doxorubicin as the chemotherapeutic agent at a perfusate dose of 320 µg/ml[27,92]. Another subsequent study demonstrated that IVLP with Doxorubicin actually increased the survival in rats with unilateral lung metastases compared to animals without any treatment and to those with IVLP without chemotherapy.[83] Melphalan was tested in the same setting, and also demonstrated that
it was effective in diminish the number of tumors compared to systemic administration of chemotherapy and IVLP without any chemotherapy[84].

IVLP was tested in a rat model of induced colorectal adenocarcinoma lung metastases, using FUDR (2-deoxy-5-fluorouridine) administered for 20 minutes, at three different doses. Compared to the systemic administration of FUDR, IVLP diminished the number of lung metastases, demonstrating that this therapy could potentially be effective in this type of cancer[93]. A very interesting study analyzed the response of Melphalan, Tumor Necrosis Factor (TNF) and the combination of both in the same type of induced lung metastases in an IVLP setting for 20 min. Melphalan demonstrated to be very effective in the treatment, whereas TNF alone did not demonstrated effective results. The combination of these drugs diminished the number of tumors but without significant advantage over the administration of Melphalan only, pointing out to the fact that TNF did not enhance the effects of Melphalan[94]. The concept to perform IVLP with two drugs was reassessed by Van Putte[95], using the same adenocarcinoma model of induced lung metastases, but at this time, the combination testes was Gemcitabine + Cisplatin, Melphalan + Cisplatin and Cisplatin + Melphalan. Compared to control groups, single agent therapy and combined therapy showed an advantage on survival rates, and the combination of drugs showed a trend to better survival with Melphalan + Gemcitabine being associated with the best overall survival at 90 days after IVLP.

Safety studies were mainly performed in large animal models. In this setting, the tolerance of the lung to a cytotoxic agent administered in an isolated perfusion circuit was analyzed by Johnston[96], where perfusion with Doxorubicin was performed in dogs
for 45 min with perfusion flows around 100 ml/min (titrated to PA pressure levels of 12-15 mmHg), using blood as the perfusate solution. Two or three weeks after the left lung perfusion, a right pneumonectomy was performed in order to check if the perfused lung was capable to maintain the animal alive. They identified perfusate levels of chemotherapy (0.5 ug/ml) that correlated to survival after right pneumonectomy, with histologic signs of mild focal pleural and sub pleural interstitial fibrosis.

The same group described that the concentration of Doxorubicin administered and the time of perfusion were factors associated with increased uptake by the lung in dogs[97]. In a tentative to augment the amount of drug administered, increased doses of Doxorubicin were associated with lung injury[98].

Baciewicz[99], studying isolated lung perfusions for 45 minutes and a follow-up of 2 months in dogs, demonstrated that perfusate concentration of Doxorubicin higher that 7 ug/ml (which corresponded to a tissue concentration of 20 ug/g of chemotherapy) was associated to lung injury and poor outcome. The histological pattern of injury at 24 hours and 72 hours post operatively were the same as those collected immediately after IVLP perfusion. This raises the question that the acute injury caused by IVLP is probably the key feature to be tackled in order to make IVLP with chemotherapeutic agents less toxic to the lung.

Using another chemotherapeutic drug (Cisplatin), Ratto treated pig lungs for four hours of IVLP followed by one hour of reperfusion, comparing stop-flow occlusion, stop-flow/out-flow occlusion and left lung perfusion with 2.5 mg/kg and with 5 mg/kg. The lung perfusion technique was associated with higher levels of chemotherapy in lung
tissue (and there was a significant difference in final tissue levels according to the perfusate levels of chemotherapy), but gas exchange and histology in the perfused lung was significantly impaired in all groups, and this was not related to the dose of chemotherapy administered neither the technique used. We may infer that, assuming that the dose of chemotherapy did not influence the toxicity found, the IVLP strategy employed might be responsible for an important fraction of the toxicity identified. There was a trend to recover of gas exchange at one hour of reperfusion and the same pattern happened with the histology (reduction of lung edema)[100] one hour after the start of blood reperfusion.

Using the same drug, a dose escalating study was done using 150 mg/m$^2$, 300 mg/m$^2$ and 300 mg/m$^2$ with hyperthermia in a large animal model of IVLP. Perfusion lasted 40 min, followed by 6 hours of reperfusion. The levels in the perfusate and in lung tissue correlated with the dose administered and IVLP was associated with impairment in functional parameters, even in the control group (IVLP without chemotherapy), compared to the sham, with a linear relationship between the dose administered and the degree of impairment found. Histological signs of lung injury were found in all animals submitted to IVLP, with alveolar edema and interstitial infiltration being more visible in the group with higher doses of chemotherapy. The control and the 150 mg/m$^2$ groups had only mild evidences of lung injury, and the sham group had less signs of injury compared to all groups[101].

The feasibility of IVLP with TNF was analyzed by Pogrebiak[92], where escalating doses were administered and the use of hyperthermia was analyzed as well. Flow rates
ranged from 200-400 ml/min, and the perfusion was performed for 60-90 minutes, using a combination of dextrose and saline as the perfusate solution. Chest X-ray demonstrated mild pulmonary congestion at the end of the perfusion (especially in cases which received hyperthermic perfusions), with improvement at the end of the first week of follow-up. Acute histologic changes like peribronchial edema and neutrophilic infiltration were seen and no long-term changes were seen at the time of elective sacrifice 6-9 months. This points to the fact that a lung injured during IVLP may recover from the insult over time. Leakage of the drug during IVLP was a complication described, with some animals presenting with hypotension and bradychardia. Pigs that presented with low systemic levels of TNF had an uncomplicated perfusion in comparison to others that presented with high levels. This group stressed the importance of a complete separation between the circuit where the drug is circulating and the systemic circulation.

Melphalan was another chemotherapeutic drug tested with IVLP. The perfusion lasted 20 minutes, followed by 24 hours of reperfusion. Small perfusion flows were used (100ml/min), and the perfusate was constituted by a colloid osmotic solution. No systemic leakage of the drug was found, and lung edema was found in the perfused groups. Tissue levels of chemotherapy showed differences between cases, meaning an heterogeneous distribution of the drug[102,103].

Gemcitabine was tested during 30 min of IVLP and one month of follow-up. A dose-response was found both in the perfusate and in the lung tissue, according to the initial levels of chemotherapy administered. Initial flows were around 500-600 ml/min, but in the animals that received chemotherapy, the PA pressure levels increased and it was
necessary to diminish the perfusion rate in order to keep these pressures within the desired range. This may suggests that the administration of chemotherapy was damaging the lung acutely, elevating the pulmonary vascular resistance (PVR) of the organ, which is the resistance that the blood flow must overcome to perfuse blood through the lungs and It is calculated based on the difference between the input pressure and the output pressure of the lung, as highlighted below:

\[
PVR = 80. \frac{\text{mean pulmonary artery pressure - mean pulmonary artery wedge pressure}}{\text{cardiac output}}
\]

An injured lung has its PVR increased, in opposition to the normal levels of PVR identified in normal lungs. Also, in this study there were no acute histologic differences between controls and chemotherapy groups, and related to long-term toxicity, peribronchial edema and interstitial cell infiltration were described, whereas no relation was found between the pathologic injury score used and the dose of chemotherapy administered.[104] Thus, IVLP without chemotherapy was comparable to IVLP with chemotherapy with lower perfusion flow rates. The injury caused by the perfusion strategy might be a reason for these findings.

In summary, the experience with large animal models of IVLP demonstrated that efficient separation between pulmonary and systemic circulation was possible and that some chemotherapeutic agents could be administered with reasonable safety.

Beyond safety and efficacy studies, other important factors were assessed, with the intention to optimize IVLP like:
Temperature of the perfusate: The use of hyperthermia with IVLP was studied with the intention to analyze if there was an enhancement of the effects of chemotherapy, assuming that a higher temperature will allow more absorption of the drugs by the organs. In an experimental setting, high temperatures (37 - 44 °C) were well tolerated during conventional IVLP [105,106] and IVLP performed bilaterally under cardiopulmonary bypass [107]. Moderate hyperthermic perfusions (41.5°C) was also tested and no differences were found in physiologic parameters in comparison to normothermic temperatures (38°C) [108]. Clinically, moderate hyperthermia was tested as well and some degree of toxicity was found [105]. In fact, it is difficult to estimate the role of hyperthermia as a source of injury considering the other factors might have an influence such as the chemotherapy type and the injury attributable to the perfusion circuit. The most recent clinical study advocates perfusions under normothermic conditions after complications such as empyema and rhabdomyolysis were reported in the cases where IVLP was associated with hyperthermia[109].

The modality of administration of perfusion (single pass x recirculating IVLP): Recirculation of the drug within a closed circuit was the approach more commonly used for IVLP. However, the drugs can also be administered as a single-pass fashion. Recirculating perfusion was associated with more lung injury than single-pass perfusion and also technically more challenging[110] but equivalent tissue levels of chemotherapy were reported with both techniques [111]. However, single-pass perfusions were associated with less uniform distribution of the perfusate in comparison to recirculation technique[96].
The importance of the direction of the perfusion flow (anterograde versus retrograde perfusion IVLP): the perfusion flow usually is administered in an anterograde fashion. However, considering that the bronchial circulation usually drains to the left pulmonary veins, IVLP performed in a retrograde way could theoretically perfuse not only the pulmonary but the bronchial areas of vascularization as well. This idea proved to be feasible and superior to conventional systemic administration of drugs [112]. At the same time, the comparison of these techniques was not conclusive. While the retrograde perfusion appears to increase the amount of chemotherapy in the hilar regions of the lung[113], a better uptake of chemotherapy in tumor cells was not found in a small animal model of induced-lung metastases[114].

Catheter-based lung perfusions: This is also were also an interesting concept explored previously, because considering the surgical complexity necessary to perform IVLP and the theoretically disadvantage that IVLP is probably a one-time event, attempts to overcome this problem were targeted with the development of catheter-based approaches for lung perfusion. In summary, a catheter is inserted inside the pulmonary artery usually percutaneously, using the femoral vein as the preferred access. One of the advantages is that this approach could in theory be repeated and also performed without a thoracotomy. On the other side, a disadvantage is that the outflow (pulmonary veins) is not controlled, which leads to a higher systemic leak of the drugs, and the consequent undesirable side effects.

Using a blood flow occlusion administration of chemotherapy to the lungs, [115]Wang showed advantages in pharmacokinetics and response to the treatment over conventional
systemic administration of chemotherapy. Also, less chemotherapy was administered in comparison to IVLP, to avoid systemic effects of the drugs, considering that there is no control of the outflow of the lung. The advantage over systemic chemotherapy was also found by Schneider [116]. Interestingly, no differences in the lung levels of Gemcitabine were found when blood-flow occlusion was compared to IVLP in rats[117] and proved to be as effective as IVLP in the management of sarcoma-induced lung metastases in rodents[118], but systemic exposition to chemotherapy was significantly increased with this therapy when compared to IVLP[119], which raise concerns about the toxicity that this modality may present. This was also demonstrated by Krueger[120]. An additional issue addressed by this group was the heterogeneous distribution of chemotherapy found with the blood-flow occlusion technique. Regarding lung toxicity, the same pattern of lung injury (histologic alterations and impairment in gas exchange of the perfused lung) was found[100] between blood flow occlusion and IVLP.

An interesting approach that combined blood-flow occlusion and control of the pulmonary veins of the lung with video assisted thoracic surgery (VATS) technique for the administration of chemotherapy was described by Demmy [121]. Despite histologic signs of lung injury, his group showed to be a feasible technique. This approach was also tested in a clinical setting, with the chemotherapy being exposed to the lung for 30 minutes in patients with oligometastastic stage 4 lung cancer, without long-term toxicity to the lungs and with reductions in the size of the lesions[122].
Clinical studies

Clinical organ perfusions were performed on 24 patients using nitrogen mustard with the intention to treat locally advanced tumors; mainly sarcomas of the extremities, but another targets were breast, lung and pelvis tumors. A perfusion circuit with a bubble oxygenator, a sigma motor pump and a reservoir (in lung perfusion, a systemic extracorporeal circuit was used as well) was used. Specifically lung perfusion was done with a left atrium (LA) to pump oxygenator to the PA, while the systemic circulation was maintained with cardiopulmonary bypass. A patient with an unresectable bronchogenic carcinoma was perfused for 30 minutes and it was described that the patient had a cough with large amounts of mucopurulent material. Seven days after the surgery, breath sounds became audible over the right chest wall and there was a progressive improvement on chest X-ray, with significant expansion of the right lung[123].

In a sequential report from the same group, 73 cases of isolate organ perfusions were done in patients with melanomas, sarcomas and carcinomas. Among this latter group, 7 perfusion were done in the lung for palliative purposes with two deaths that happened in the early postoperative period.[75]. The same group published their results of 411 perfusions in 350 patients between June 1957 and March 1962, but only 4 lung perfusions were performed, and they concluded that the morbidity and mortality in this group was high, therefore isolated perfusions were not indicated for lung tumors. [77]. Melanoma and soft tissue sarcomas of the limbs presented the better results with this strategy[78].
In a safety study, three patients with unresectable lung metastases from sarcoma were submitted to IVLP with Doxorubicin, and the levels of chemotherapy in the tumors were lower compared to normal lung tissue, but as time of perfusion increased, higher levels of drugs were found in lung tissue.[97]

The perfusion of the lung was studied again by Johnston[124], describing the use of this approach in patients with unresectable lung metastases from metastatic sarcoma or metastatic bronchoalveolar carcinoma. Three patients were submitted to unilateral perfusion and five patients to bilateral perfusions, for 45 minutes, using a flow of 400-680 ml/min for the unilateral perfusions and higher flows for the cases done under cardiopulmonary bypass, which was calculated according to the PA pressure levels (which were maintained between 14-18 mmHg). Of note, the authors described that physiologic flow rates would not be possible to obtain in single lung perfusion without exceeding normal PA pressures. Doxorubicin was the drug used in 6 patients and two received Cisplatin. Despite the variability found, tissue levels of drugs tend to increase with higher doses administered, and optimal separation between systemic and pulmonary circulation was achieved, however no partial or complete clinical response was found and two major postoperative complications were described.

A phase-1 study using TNF was performed in patients with unresectable pulmonary metastases from sarcoma[102], melanoma and others histologies, in 15 patients. During 90 minutes, IVLP was performed using saline solution as the perfusate and perfusion flow rates were controlled according to the PA pressure levels (below 20-30mmHg), with control of the bronchial circulation during isolated lung perfusion. Tissue levels
increased according to increase in the dose administered. A mild bronchorrhrea from the perfused side was described during most of the perfusions, ceasing in the early postoperative period and could be attributable as an evidence of lung injury during IVLP. Three partial responses were found, but they were short lived. Chest X-ray immediately after the perfusion revealed asymmetric infiltrates and minimal effusion, which resolved over time. One late complication was described (lung abscess).

The used of the perfusion in patients with resectable lung metastases, as an intraoperative measure to diminish the odds of local recurrence after lung metastasectomy, was first described by Ratto[125]. In his study, 6 patients with metastatic sarcoma were submitted to IVLP with Cisplatin 200mg/m² during 60 minutes, followed by lung metastasectomy. Perfusate solution was a combination of Haemmacell, ringer solution and heparin, and was oxygenated to prevent vasoconstriction and the outflow was drained by gravity. Flows were adjusted to a PA pressure below 35 mmHg, resulting in rates of 200-280 ml/min. No attempts to control bronchial circulation were done and after the perfusion, an increase of 50% of the priming volume was noted in the reservoir of the perfusion circuit. Acute toxicity was described in 2 cases, with interstitial and alveolar edema on chest X-ray. Systemic toxicity was not found and no late morbidity was described. The pulmonary function tests showed impairment at 10 and 30 days postoperative, returning to baseline levels at 6 months. No histologic damage to the lung parenchyma was described. With the intention to treat micrometastatic disease, only tumors smaller than 0.1 cm were used for pharmacokinetic purposes and in these nodules, the dose of chemotherapy was similar to the dose found in normal lung tissue. This information is
very important because smaller tumors may absorb more chemotherapy in comparison to larger lung metastases, constituting a better target to IVLP.

Burt described the experience using IVLP in a phase-1 study for patients with unresectable lung metastases in 8 patients with metastatic sarcoma using Doxorubicin at a dose of 40mg/m² in the majority of patients (Figure 7).

![Figure 7 – IVLP technique used in clinical setting](image)

The perfusion lasted 20 minutes using a colloid solution as the perfusate and the flow rates used were 300-500 ml/min. Bronchial arteries were occluded with a clip. A correlation between the dose administered and normal and tumor tissue levels were found, but the tumor tissue absorbed less chemotherapy compared to the normal lung.
Heterogeneous levels of chemotherapy were described both lung and tumor tissue. Only one patient received a dose of 80 mg/m² of Doxorubicin, and this patient had a major complication related to the perfusion. The remaining of patients all had some degree of pulmonary toxicity and with a median follow-up of 11 months, no partial or complete responses were found[126].

Hyperthermic lung perfusions were studies by Schroder[105] in 4 patients with resectable (n=3) and unresectable (n=1) lung metastases from sarcomas. Whenever applied, lung metastasectomy was done before IVLP and perfusion with Cisplatin 70 mg/m² for 20-30 minutes was performed, with a flow of 300-500 ml/min, which was adjusted to the PA pressure levels, while the perfusate temperature was kept at 41 °C. No evidence of chemotherapy-related systemic toxicity was described, and patients had pulmonary toxicity mainly as non-cardiogenic lung edema and ischemic mucosal changes in the bronchus of the treated lungs. Within the next 12 weeks, all these complications gradually improved and at 12 months of follow up, 75% of patients were alive and the only patient who died was because of cerebral metastases, without evidence of local recurrence.

In another study phase-1 study, IVLP with Doxorubicin was performed in 15 patients with unresectable lung metastases. The perfusion lasted 20 minutes and higher levels of chemotherapy were found in lung tissue in comparison to intra-tumoral levels, with a wide range of variation in the levels of chemotherapy was found (lung levels the median 125 µg/g tissue, range 9.4 -193 µg/g; tumor levels median 58 µg/g tissue, range 9.5 -117 µg/g). Systemic toxicity was not found and two patients perfused with a dose of
75mg/m² of chemotherapy developed severe pulmonary toxicity. Operative mortality was 20% and late toxicity included a 40% decrease in ventilation and perfusion of the treated lung. Median survival was 19 months and five patients had regression or stabilization of the disease[127].

A phase-1 multicenter study using this approach had their results reported in 2004 [128], where 16 patients with resectable lung metastases from primary tumors like colorectal, renal carcinomas and sarcomas, using increasing doses of Melphalan as the chemotherapeutic agent. Metastasectomy was performed after IVLP and the perfusion lasted 30 minutes, with a flow rate of 0.7 l/m² of body surface, adjusted to a PA pressure level below 30 mmHg. Perfusate solution used was a mixed of a colloid solution + Heparin. Bronchial circulation was controlled by snare the bronchus of the perfused lung. Operative mortality was 0% and no systemic toxicity was described. However, important toxicity was found in 2 of 3 patients perfused with a dose of 60 mg at normothermic state. After a mean follow-up of 14 months, all patients were alive, but recurrent metastatic disease occurred in 7 patients (4 outside the lung, 2 in the nonperfused lung and 1 in the perfused lung). The disease free interval for the lung recurrences was 9 months (range 7-11 months).

An extension trial from the same group focused on pharmacokinetics was reported by Grootenboers [129] where 7 patients were submitted to IVLP with doses of 15 and 45 mg of Melphalan under hyperthermic conditions. A relevant variability between levels of chemotherapy in perfusate, lung and tumor tissues was found and a correlation between the perfusate levels and the lung tissue levels was found.
The survival rates of the phase-1 trial were published in 2010[130], where 29 procedures were performed in 23 patients. As previously mentioned, Melphalan was given at 45 mg under normothermic temperature. One patient lost follow-up, and after a median of 62 months, 6 of 23 patients were alive and without disease. From the 16 patients who presented with recurrent disease, 9 had intrathoracic recurrence only (3 in the perfused lung only, 2 in the perfused lung with loco-regional metastases, 4 in the non perfused lung), and the 23 patients (47.8%) of the patients were alive at the end of follow-up, with 6 without disease, with a 5-year survival of 54.8% and 5-year DFI of 27.5%. Number of metastases, type of tumor and DFI did not influence the survival. Lung function stabilizes and slightly recovers after a decrease after IVLP, with a stable function 6 months after. No major long-term toxicity was found.

The description of these studies is highlighted in Table 2. Lung injury was the main complication reported, and lung edema was a frequent finding after the procedure. The questionable efficacy is also highlighted, with a small number of patients presenting with partial responses, and even this response was typically short-lived. Many drugs were tested and the same can be mentioned about perfusate solutions, time of perfusion and type of lung metastases (resectable versus unresectable). A more detailed analysis of the results lead to us to infer that the lung usually recovers over time, considering the fact that the clinical signs usually resolve weeks after IVLP. Interestingly, most of the toxicity was attributed to the chemotherapy itself, but other factors may contribute to this as well, like the strategy used to perfuse the lung in situ.
The clinical studies demonstrated that lung tissue levels of chemotherapy correlated with the dosage administered. However, a heterogeneous distribution in final lung levels was found in most of the studies. It is important to note that lung metastasectomy with IVLP could be done with comparable results to conventional lung metastasectomy regarding post-operative quality of life scores[131], thus, a technique which minimizes lung injury, allows a better distribution of chemotherapy in lung tissue would be an important achievement to this field.

### 1.2.4 IVLP - A critic of studies performed to date

Phase-1 clinical studies using IVLP as a potential treatment to lung metastases almost uniformly described acute and chronic lung toxicities as the main limitation factors. In
this setting, the chemotherapeutic drugs used in the perfusate were appointed as the main reason for these patterns of injury (Figure 8).

**Figure 8 – Lung injury reported in IVLP.** A patient before (A) and 2 weeks after left lung IVLP (B) with Doxorubicin at a dose of 80 mg/m³, where severe signs of acute lung injury can be found in the perfused lung[126].

Toxicity caused by the perfusion circuit should not be forgotten and it is important to note that it was unclear from many of these studies whether the perfusion procedure itself or the chemotherapy was the major responsible for the lung injury. In theory, protective environment for the safe delivery of chemotherapy may minimize the deleterious effects of these drugs in normal lung parenchyma. Thus, a new strategy where the perfusion circuit and the perfusion/ventilator strategy that minimize injury to IVLP needs to be established.

The anticancer efficacy of IVLP has also been questionable and several reasons can be hypothesized:
1. Single agent was applied: most of the current chemotherapeutic schemes used for the management of cancers are a combination of drugs, like the FOLFOX scheme for colorectal cancer or the AIM 75/9 for metastatic sarcoma. IVLP was mainly done with single drugs previously, however a study in small animals demonstrated that a combination of drugs is more effective in this setting[95]. Large animal studies and clinical series of IVLP did not explore this concept with a mixed scheme of chemotherapeutic drugs.

2. Lack of tumor-selectivity limits increasing drug uptake into tumor tissue without increasing uptake in healthy tissue: the majority of the clinical and experimental studies showed that normal lung tissue achieved more tissue concentration of chemotherapy when compared to the tumor tissue and this was always a concern, considering the oncological purpose of this approach. Increments in the chemotherapy dose with the intention to elevate the levels of drugs in tumors were invariably associated in dose-related lung injury.

3. Large variations in final lung tissue levels of chemotherapy: The majority of studies done so far demonstrated heterogeneous distribution of chemotherapy in lung tissue.

4. Selection of patients: Patients submitted to IVLP were mainly with unresectable metastatic disease. To target the micrometastatic disease can potentially enhance the effects of IVLP. Smaller tumors may be able to absorb more chemotherapy compared to larger lesions as demonstrated in a clinical setting by Ratto in patients submitted to IVLP with resectable lung metastases, where equivalent
levels of chemotherapy between normal and tumor tissue were found when
nodules with dimensions smaller than one centimeter were analyzed[125] and in
an experimental setting, where an inverse correlation[132] between tumor levels
of chemotherapy and size of nodules were studied.

5. The duration of the perfusion: very brief IVLP time was explored in the majority
of the studies, ranging from 20-40 minutes, which in fact means that the exposure
time of the lung to the drugs was usually short. An increase in the perfusion time
correlates with elevated levels of chemotherapy in lung tissue[14,97,133], and
this needs to be explored.

A reliable model for proper delivery of chemotherapy to the lungs where high levels of
chemotherapy can be found in lung tissue, with minimal systemic exposure and with
minimal but permissive toxicity to the lungs is needed.

Ideally, according to Putnam, lung ischemia and endothelial injury must be minimized
during IVLP and chemotherapy concentrations must be optimized for the tumor. The
concentration of the chemotherapeutic agent and the circulation time during IVLP must
be sufficient to allow effective exposure of the chemotherapeutic agent to the lung
metastases [134].
1.2.5 The Ex Vivo Lung Perfusion strategy – a platform to develop an optimized IVLP strategy

Ex Vivo Lung Perfusion (EVLP) is a technique where lungs are perfused outside the body, and due to a specific perfusion and ventilatory strategy, have their function optimized. However, this concept is not new and served as a platform to study the function of lungs in the past[135,136], but the potential use as a strategy that could maintain lung function resulted in injury of the organ[137].

One of the keys features that made EVLP possible was the development of a specific solution (Steen solution) that allowed the perfusion of lungs without the development of lung edema for non-heart beating donors [138]. This group reported that an improvement in the pulmonary function was possible in human donors rejected for clinical transplantation [139], with EVLP being performed for short periods of time (1-2 hours). However, the attempts to perform EVLP for extended periods of time were elusive, and invariably associated with lung edema [140,141].

The long-term EVLP perfusion strategy was developed by Cypel[142,143], and, in addition to the work previously done, several strategies were implemented by this group, like the use of physiologic perfusion flows, the focus on interactions between ventilation and perfusion, the use of a positive left atrial strategy and the perfusion under normal PA pressure levels (Figure 9).

The outcomes reported were so dramatic that even lungs that initially were not suitable for clinical transplantation could have their function optimized, becoming organs that
could be effectively transplanted, with equivalent results to lungs considered as “ideal” for clinical transplantation[144].

**Figure 9 – The Toronto EVLP strategy.** Lungs are perfused inside a sterile chamber, outside the body. The perfusate leaves the lungs through the LA (red), then goes to a reservoir and sequentially a centrifugal pump send the flow to a membrane oxygenator that receives a mixture of gas whose function is to deoxygenate the perfusate, mimicking normal lung physiology. The perfusate deoxygenated (blue) is then directed to a leukocyte filter and returns to the lungs through the pulmonary arteries.

This group extended the potential applications of EVLP to enhance gene therapy delivery to donor lungs[145]. Hence, the validation of this technique in a clinical setting was published by the same group, demonstrating the safety and efficacy of EVLP as a new tool to accurately access and improve the function of lungs initially deemed not suitable for clinical transplantation[146].

The EVLP platform provided several principles that were used for the IVLP project described here:
• Acellular solution with an optimal colloid osmotic pressure
• Protective flow to perfuse the lungs
• Protective ventilator strategy
• Positive left atrial pressure
• PA pressure within the normal range
• Normothermia

In the IVLP setting, lung injury described was invariably associated with the effects of the chemotherapy itself. However the perfusion/ventilatory strategy may be a source of lung injury that was attributed to the chemotherapy only. In this setting, a strategy that potentially minimizes lung injury, allowing a more effective delivery of drugs, may establish an approach that has the potential to help a population of patients who unfortunately does not have many effective options for their treatment.
2 Rationale, Hypothesis, Objectives and Study Design
2.1 **Rationale**

IVLP can potentially be used to improve the outcomes of patients with lung metastases. Previous attempts to perform IVLP presented variable lung toxicity and questionable efficacy, hindering the broad application of this technique. Based on a model designed to improve the function of lungs that were initially not suitable for clinical transplantation, we aim to develop a protective strategy to administer high-dose chemotherapy to the lungs in a safe manner. A modified IVLP strategy that minimizes perfusion-related injury, allows an extended perfusion time and optimize the chemotherapy levels in lung tissue can be a major step towards the wide spread clinical application of this technique.

2.2 **Hypothesis**

- A novel IVLP technique based on the Ex Vivo Lung Perfusion model may allow perfusion with minimal lung injury, for an extended period of time.

- A novel IVLP technique based on the Ex Vivo Lung Perfusion model may allow a safe delivery of high-dose sarcoma based chemotherapy to the lung.

2.3 **Objectives**

1. The development of a strategy for IVLP, which allows for an extended perfusion time, without inducing significant acute lung injury.
2. Analysis of the safety and pharmacokinetics of administration of high-dose sarcoma
based chemotherapy to the lungs during IVLP.

2.4 Study Design

2.4.1 Phase I – Aims

1) Development of a large animal model of IVLP incorporating a protective strategy and enabling an extended perfusion time.

2) Assessment of the distribution of the perfusate in the lung parenchyma, using India ink as a marker agent.

Endpoints:

1) Lung function of the perfused lung before, during IVLP and after reperfusion

2) Histologic assessment of acute lung injury

3) Chest X-ray before IVLP and at the end of 4h IVLP + 4h reperfusion
4) Macroscopic assessment of the distribution of India ink in lung tissue

2.4.2 Phase II – Aims

1) Determine if the standard dose of chemotherapy (Doxorubicin 75mg/m²) used in a clinical setting can be safely administered via our IVLP strategy.

2) Determine if a mixed scheme of chemotherapy (Doxorubicin 75 mg/m² + Ifosfamide 6g/m²) can be safely administered using the proposed IVLP strategy.

3) Perform a dose-escalating study using Doxorubicin as the chemotherapeutic drug, in order to find the limits of toxicity of the IVLP strategy.
4) Determine the importance of IVLP duration, comparing IVLP for 2h versus 4h

Endpoints:

1) Lung function of the perfused lung before, during IVLP and after reperfusion.

2) Histological assessment of acute lung injury.

3) Lung inflammation profile: cytokine levels in lung tissue, comparison of baseline (before IVLP) and reperfusion samples.

4) Measurement of apoptosis in lung tissue - assessed at the end of reperfusion.

5) Pharmacokinetics of the chemotherapy in lung tissue, perfusate solution and systemic circulation.

6) Determine the influence of IVLP time in relation to chemotherapy tissue levels at the end of reperfusion.
Note: Two additional endpoints were analyzed, which were not directly related to the Phase II, but were important in relation to the studies with chemotherapy, and may have influenced the results:

7) Determine the influence of the bronchial circulation in the final tissue levels of chemotherapy.

8) Determine if the chemotherapy was not bounded to the perfusion circuit during EVLP.

2.4.3 Phase III – Aims

1) Evaluate whether the IVLP strategy developed is reproducible in human lungs subjected to ex vivo lung perfusion with chemotherapy.

Endpoints:

1) Lung physiology during EVLP

Phase III study design:
3 Methods
3.1 Animals

Yorkshire pigs weighing an average of 35-42 kg were used for all experiments. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care of Laboratory Animals” published by the National Institutes of Health. The Animal Care Committee of the Toronto General Research Institute approved this study.

3.2 Human lungs

Human lungs declined for clinical transplantation were used to study the effects of high-dose chemotherapy during EVLP and to study pharmacokinetics. Considering that the sensitivity of the lung to a drug might vary between species, human lung studies will provide critically important information. Only lungs from donors who specified that their organs could be used for research projects were used for this study. This is currently an active model in our laboratory and is approved by UHN CAPCR and Ontario Trillium Gift of Life (OPO).

3.3 Experimental Strategy

Pigs were initially sedated with ketamine 20 mg/kg + midazolam 0.3 mg/kg + atropine 0.04 mg/kg, then inhalator anesthesia was started with Isoflurane 5% and maintained
with a combination of intravenous Propofol (5-8mg/kg/h) and fentanyl citrate (2-20 mg/kg/h). After induction of general anesthesia, a tracheostomy was performed and a 19 French (Fr) left sided double lumen endotracheal tube was positioned to allow the measurement of the airways mechanics of the perfused lung throughout the procedure. During the experiments performed with chemotherapy, a single lumen endotracheal tube was used to avoid discrepancies in the ventilation-perfusion ratio, which could influence the distribution of chemotherapy in lung tissue due to the mismatches of ventilation/perfusion that might happen in poor-ventilated areas[6]. Ventilation was maintained during the entire experiment with an ICU ventilator (Servo-1, Maquet Critical Care, Solna, Sweden) according to the ventilator parameters listed in Table 3.

### Ventilatory parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tidal volume</td>
<td>8 ml/kg</td>
</tr>
<tr>
<td>PEEP</td>
<td>5 cmH₂O</td>
</tr>
<tr>
<td>Respiratory rate</td>
<td>16 breaths/minute</td>
</tr>
<tr>
<td>FiO₂</td>
<td>50%</td>
</tr>
</tbody>
</table>

*Table 3- Ventilatory strategy used for IVLP.* PEEP, positive end-expiratory pressure; FiO₂, fraction inspired of oxygen.

A right neck dissection was performed to isolate and cannulate the right carotid artery (for continuous assessment of systemic pressure) and internal jugular vein (as an accessory intravenous line). Animals were subjected to a four hour period of left lung IVLP followed by a single pass gravity-driven wash-out and additional four hours of reperfusion.
The study design consisted in four hours of left lung IVLP, a single-pass washout and additional four hours of reperfusion:

At the end of the reperfusion, animals were sacrificed by exsanguination or by a lethal injection of sodium pentobarbital (15 ml – 240 mg/ml).

### 3.4 IVLP Procedure

A left postero-lateral thoracotomy was performed and the left azygous vein was ligated over the descending aorta, with dissection carried towards the left PA, which was mobilized in the entire circumference and encircled. Proper length of the PA is necessary for the insertion of the cannula, and mobilization must be performed from the root of the left PA until the site where the left PA starts the division in branches. Next, dissection was carried down towards the atrium. In this area, the dissection of the azygous vein that is important because this vessel is over the left upper vein, hiding it. With gentle dissection, it is possible to release the posterior surface of the left azygous vein from the anterior surface of the left upper vein. The dissection of the left lower vein is easy considering the size of this vessel. However, in this part of the surgery, identification of the mediastinal branch, which in fact drains the caudate lobe, is critical because this lobe
is not a part of the left lung and should not be included in the perfusion. Full mobilization of the upper and lower left pulmonary veins can be seen in Figure 10.

![Image of left atrium with upper and lower veins identified and encircled.](image)

**Figure 10 – Left atrium exposed, with upper and lower veins identified and encircled.** For this purpose, a dissection plane was created between the left azygous vein and the LA. Proper identification of the mediastinal venous branch is very important as this facilitates the positioning of the vascular clamp in the LA.

Lastly, we dissected and isolated the left main bronchus circumferentially, directing the dissection towards the tracheal carina. At this point, it is important to remove the lymph nodes that were located in the subcarinal area. Also it is important to strip the peribronchial tissue with the intention to diminish bronchial circulation leakage to the isolated lung circuit (Figure 11). To do this, we should choose a point close to the hilum of the left lung and in that area, the left main bronchus should be cleaned in its entirety.
circumference, allowing a clean visualization of the bronchial wall. This is a very important step because major bronchial arteries can be not completely visualized and once IVLP is started, a suboptimal control of this circulation may lead to spillage of systemic blood to the perfusion circuit, resulting in hemodynamic instability for the pig during the procedure.

**Figure 11 - Isolation of bronchial circulation.** The left main bronchus is dissected circumferentially and completely stripped from the surrounding tissues, in order to diminish the bronchial circulation to the left lung.

After dissection of these structures, two concentrically purse string sutures were placed in the left PA and one purse string suture in each left pulmonary vein. Heparin 5,000 IU (Sandoz, Quebec City, Canada) was administered through a central venous line and a cannula (Cardioplegia Cannula Anterograde, 13 Fr Maquet Cardiopulmonary, Rastatt, Germany) was inserted in the PA and snared. A right angle cannula (Venous catheter
OD 12 Fr, Maquet Cardiopulmonary, Rastatt, Germany) was then inserted in the upper and in the lower left pulmonary veins (Figure 1). The venous cannulas were inserted as close as possible to junction of the pulmonary veins to the LA, to prevent selective cannulation of venous branches. To monitor the pulmonary and the LA pressures, we used 5 Fr pediatric feeding tubes, which were inserted in the PA and in the left lower vein, respectively, and connected to a monitor (SurgiVet, Smiths Medical, MA). Prior to IVLP initiation, PA and LA vascular clamps were inserted to isolate the left lung from the systemic circulation.

3.5 IVLP circuit

The perfusate was circulated by a centrifugal pump (Revolution, Sorin Group USA Inc, Arvada, Colorado) passing through a leukocyte depletion filter (LG-6 Leukocyte Reduction Filter, Pall Corporation, NY) and a membrane gas exchanger (Quadrox-i Oxygenator System, Maquet Cardiopulmonary AG, Germany) before enters the left lung through the PA. A filtered gas line for the gas exchange membrane was connected to an H-size specialty gas mixture of Oxygen (6%), Carbon Dioxide (8%) and Nitrogen (86%) (Praxair, Mississauga, Canada). This mixture will deoxygenate the perfusate and provides carbon dioxide to the inflow maintaining PCO$_2$ between 35 and 45 mmHg (the gas was started at 1 liter / min and was adjusted in order to have PCO$_2$ levels as mentioned). These levels, whenever not within the proper range, may lead to endothelial injury and impairment of alveolar fluid re-absorption[147]. A Heat - exchanger (Sarns, Ann Arbor, MI) was connected to the membrane gas exchanger in order to maintain the
perfusate at 37 °C of temperature. PA flow was controlled by the centrifugal pump and measured using an electromagnetic flow meter (Sorin Group, Munchen, Germany). The outflow perfusate returns through the left atrial cannulas to a hard-shell reservoir (Maquet Cardiopulmonary AG, Germany). The height of the reservoir controls the LA pressure. The system is described in Figure 12.

**Figure 12 – IVLP strategy developed.** The perfusate is circulated by a centrifugal pump passing through a membrane gas exchanger, which receives a combination of gas (oxygen 6%, carbon dioxide 8% and nitrogen 86%) to deoxygenate the perfusate, and provide CO₂ for the inflow. Flow is then directed to a leukocyte filter and enters the lung through the left PA. The temperature of the perfusate is maintained with the aid of a heat exchanger, which is connected to the membrane gas exchanger. PA flow is controlled by the centrifugal pump and measured using an electromagnetic flow meter. The outflow perfusate returns through the left pulmonary veins to a hard-shell reservoir, and the height of this reservoir is adjusted in order to have the drainage pressure within the appropriate range. Catheters in the left PA and in the left lower vein measure, respectively, PA and LA pressures. A standard ICU-type ventilator provides ventilation to the lungs.
3.6 Priming the circuit

The reservoir of the circuit was primed with 1.2 liters of Steen solution (XVivo Perfusion, Göteborg, Sweden), which is a buffered dextran containing an extracellular solution with an optimal colloid osmotic pressure, developed for extracorporeal perfusion of lungs. The components of the Steen solution are described in Table 4. In addition to this, 500 mg of Methyl prednisolone (Solu-medrol, Pfizer, Kirkland, Canada), 1g of Cefazolin (Pharmaceutical Partners of Canada Inc, Richmond Hill, Canada) and 5,000 IU of Heparin were added to the perfusate.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum albumin</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride</td>
<td></td>
</tr>
<tr>
<td>Dextran 40</td>
<td></td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>Sodium chloride</td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td></td>
</tr>
<tr>
<td>Potassium chloride</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Composition of Steen solution.

3.7 Perfusion Strategy

Perfusion was started at 37 °C of temperature, with continuous measurement of the systemic, PA and LA pressures, with a flow established according to the estimated CO of
the animal. These parameters were modified from the EVLP strategy. We used 16% of estimated CO to perfuse the left lung only.

The levels of PA pressures are obtained and adjustments in the height of reservoir of the perfusion circuit are done, in order to keep the LA pressure between 3-5 mmHg.

Perfusion parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>16% of cardiac output</td>
</tr>
<tr>
<td>Pulmonary artery pressure</td>
<td>10-15 mmHg</td>
</tr>
<tr>
<td>Left atrium pressure</td>
<td>3-5 mmHg</td>
</tr>
<tr>
<td>Temperature of perfusate</td>
<td>37°C Celsius</td>
</tr>
</tbody>
</table>

Table 5 – Perfusion strategy developed for the IVLP technique.

The rationale for this flow rate is that, in the EVLP protocol, we perfuse both lungs with 40% of the calculated CO. From this value, approximately 60% (which means 24% of the entire CO) of the flow is directed to the right lung and 40% (meaning 16% of the CO) to the left lung. Using that calculation, usual flows are 450 to 550 ml/min depending on animal weight and estimated CO.
In order to reach these rates, we used a strategy to gradually increase the perfusion flows in a three step-way, starting with 30% of the calculated flow for 10 minutes, then moving to 60% for the next 10 minutes and finally to 100% (full IVLP flow).

3.8 Ventilation strategy for measurement of left lung physiology

During the entire procedure, both lungs were ventilated, but specifically during IVLP, the right lung performed oxygenation of the animal exclusively. Using a double lumen endotracheal tube, we temporarily excluded the right lung in order to assess pulmonary mechanics of the left lung only. This was performed every hour during IVLP. After this measurement, ventilation for both lungs was restarted, and a recruitment maneuver to a Peak airway pressure (Pawp) of 25 cm H₂O was done, to fully re-expand the right lung. It is necessary to control the oxygenation level of the animal because hypoxia may appear promptly during right lung deflation.
3.9 **Collection of data**

In every experiment, proper identification of the animal was done. Additionally, the weight of the animal was reported and the settings were calculated according to this information, like the CO₂, the tidal volume, the perfusion flow and the dose of chemotherapy to be administered in the specific experiment.

Parameters were registered before IVLP (baseline) and after IVLP with hourly assessments thereafter. We measured airway dynamics (Pawp: peak airway pressure, Pplat: peak plateau pressure, Cdyn: dynamic compliance, Cstat: static compliance) as well as the inflow pressure (PAP: PA pressure) and the outflow pressure (PLA: LA pressure). Establishing the inflow and the outflow pressure, we were able to generate the vascular resistance of the perfused lung (PVR: pulmonary vascular resistance). A reliable way to assess the status of the animal during the experiment was done with the arterial blood gases collected from the systemic circulation (pH, PCO₂ and PO₂ syst). The pH, PCO₂ and PO₂ that were collected from the outflow (LA) during IVLP and from the left lower vein during reperfusion were called “post lung” and served as a measurement of the lung function. The pH, PCO₂ and PO₂ pre lung that were collected from the inflow (PA) were named as “pre lung” and collected only during IVLP to assess the level of gas administered for optimal deoxygenation of the perfusate solution.

The data collected from each experiment were registered in a flow sheet like the one described below (Figure 13).
Figure 13 – Collection of data for IVLP. Datasheet developed for a careful assessment of the parameters during the experiments.
3.10 Terminating IVLP and reperfusion phase

After four hours of IVLP, venous cannulas were removed and a washout period was done in an anterograde fashion with 500 ml of a low-potassium dextran solution (Perfadex; XVivo Perfusion, Göteborg, Sweden), positioned 30 cm above the animal, allowing a passive drainage of the effluent through the left pulmonary veins by gravity. This washout procedure lasted 10-15 min. The purse string sutures in the pulmonary veins were tied and the atrial clamp was released. The next step is the careful release of the LA and PA clamps. A review of the operative field was done, and any possible source of bleeding was identified and fixed. The pressure monitor catheters were left in place for measurement of PA pressure in the reperfusion period and to collect samples from the lower lobe vein.

3.11 Lung function assessments

Lung function was assessed before IVLP, hourly during IVLP as well as during reperfusion. We evaluated gas exchange function (PaO$_2$/FiO$_2$) through samples collected from the left lower vein (before IVLP and during reperfusion) and from the outflow of the perfusion circuit (during IVLP), airway dynamics (airway peak and plateau pressures), lung compliance (static and dynamic), and pulmonary vascular resistance {((PA pressure-LA pressure) x 80 / PA flow [dynes/sec/cm$^5$])}. Gas Exchange, pulmonary vascular resistance, peak airway pressure and dynamic compliance were recorded from both lungs, and also from the perfused lung only. During the development of the large animal model,
a double lumen endotracheal tube was used, which allowed an assessment of the airway dynamics during the entire experiment.

3.12 Histology

Lung tissue biopsies were collected from the periphery of the lung before and after IVLP, and 4h after reperfusion. Tissue samples were fixed in 10% buffered formalin for 48 hours, then transferred to 70% alcohol solution, embedded in paraffin, sectioned at 5-µm thickness, stained by hematoxylin and eosin (H&E), and examined for pathologic changes under light microscopy. Pathologic acute injury score used the following criteria: air space hemorrhage (presence of red blood cells in the alveoli), vascular congestion (>75% of alveolar septum occupied with red blood cells), fibrin in the alveoli, and presence of infiltrating white blood cells.

These criteria were graded on a scale ranging from normal appearance (0%), mild (10%), moderate (10-50%) and severe (>50%) abnormalities and scored from 0 to 3, respectively[148]. The assessment of the injury scores was performed by Dr. David Hwang, in a blindly fashion.
3.13 Radiologic Assessment

Radiologic assessment of the perfused lung, comparing the images before IVLP and at the end of the experiment, was used to assess possible signs of acute lung injury such as pulmonary infiltrates.

3.14 Administration of chemotherapy

The body surface area (BSA) of the pig was calculated according to Brody’s formula[149]:

\[ \text{BSA (m}^2\text{): } 0.09 \times \text{body weight (kg)}^{0.633} \]

The doses of chemotherapy administered in these experiments were chosen according to the standard doses of Doxorubicin and Ifosfamide which should be administered in a clinical setting, using the regimen AIM 75/9[150].

The regimen is described below:

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose and Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>75 mg/m²</td>
</tr>
<tr>
<td>Ifosfamide</td>
<td>6 g/m²</td>
</tr>
</tbody>
</table>
In a clinical setting, each cycle of AIM 75/9 lasts 21 days, and drugs are given at the day 1. One of the most common side effects of Ifosfamide is hemorrhagic cystitis, and when this drug is administered systemically, an uroprotectant drug called Mesna should be added to this regimen in order to avoid this specific problem. In the IVLP setting, we decided not to include this drug in the regimen, considering that systemic exposition to the drugs should be minimal.

Hence, the chemotherapeutic drugs used in our study were administered according to the formulations described below:

Doxorubicin (02mg/ml, each vial with 100 ml) was administered directly inside the reservoir, during 3-5 minutes, as a bolus after full perfusion flow was established.

Ifosfamide (1g/vial, powder, need to be diluted with 20 ml of sterile water) administered to the perfusion circuit immediately after full flow was established.

3.15 Pharmacokinetics of drugs

The analysis of the pharmacokinetic properties of the drugs during IVLP and reperfusion was assessed in perfusate collected from the perfusion circuit during IVLP, from serum during IVLP and reperfusion and from lung tissue at the end of reperfusion. The collection of each sample is described below.

**Perfusate:** Samples were collected from the perfusion circuit 10 minutes after injection of the chemotherapy in the perfusion circuit and 1,2,3 and 4 hours sequentially. These
samples were immediately put on liquid nitrogen and later on a -80 °C freezer for future analysis.

**Serum:** Samples were collected from the systemic circulation at the end of IVLP and 2 hours after blood reperfusion had started. These samples were put on ice for 90 minutes until clotted. Then, centrifugation was performed for 15 min at 4 °C with 15000x g. The serum was separated, snap-frozen in liquid nitrogen and stored in a -80 °C freezer for future analysis.

**Lung tissue:** biopsies were collected from three different areas of the perfused lung (upper, middle, lower parts) at the end of the experiment, immediately snap-frozen on liquid nitrogen for later storage at -80 C freezers. These samples were used for measurement of drug levels and also for inflammatory profile.

### 3.16 Doxorubicin analysis in samples

Doxorubicin levels in perfusate, serum and lung tissue were assessed with High Performance Liquid Chromatography (HPLC). The sensitivity for detection of Doxorubicin using HPLC is 2 ng.

Doxorubicin hydrochloride (Adriamycin) was supplied as solution ready to use (2 mg/ml) from Pfizer Enterprises (Canada) and Daunorubicin (used as internal standard), 1-heptanesulfonic acid, zinc sulfate, potassium phosphate (KH2PO4) were purchased from Sigma. Acetonitrile of HPLC grade, acetone, hydrochloric acid (HCl) and acetic
acid (glacial) were obtained from Caledon (Canada). Purified water was obtained from a milli-Q Advantage A10 apparatus (Millipore, Bedford, USA).

Sample extraction/preparations and HPLC analysis were performed using a modified method published by Kummerle et al [131,151].

The liquid chromatography system consisted of an Ultimate 3000 pump (Dionex), an Ultimate 3000 automated sample injector (Dionex) provided with a 250 µL sample loop and a sample compartment temperature set up at 4°C, an Ultimate 3000 thermostated column compartment and a RF 200 Fluorometer (Dionex) with excitation and emission wavelengths set at 482 and 550 nm, respectively.

Chromatographic separation were carried out at room temperature using a ChromCart cartridge column (125 x 4 mm ID, 5 µm) Nucleosil 100 C18 AB (Macherey-Nagel) equipped with a guard column (8 x 3 mm ID, 5 µm) Nucleosil 100 C6H5 (Phenyl) end-capped (Macherey-Nagel).

The mobile phase was delivered at 1 mL/minute and consisted of acetonitrile and 1-heptanesulfonic acid (0.2%) pH 4.0 (with 0.1 N acetic acid), with the following step-wise gradient elution program: acetonitrile/1-heptanesulfonic acid (0.2%) pH 4.0 15/85 at 0 min, then 15/85 to 50/50 (from 0 to 20 min), then 50/50 to 100/0 (from 20 to 22 min) followed by 100/0 to 15/85 (from 22 to 24 min) and finally keeping 15/85 for re-equilibration from 24 to 26 min.

Chromatographic data acquisition and reprocessing was performed using Chromeleon software version 6.80 (Dionex).
The column was initially equilibrated with mobile phase (acetonitrile/1-heptanesulfonic acid (0.2%) pH 4.0) 15/85 to get a stable baseline followed by 2 injections of a standard mixture containing Doxorubicin and Daunorubicin (used as internal standard).

For each set of experiment (serum and perfusate samples or lung tissues samples), a Doxorubicin standard curve (spiked with Daunorubicin: 200 ng) was generated at 0, 10, 50, 100, 250, 500 and 1000 ng in 100µL final volume. Quantitative analysis of Doxorubicin was performed by using the IS method. Calibration curves were obtained by linear weighted (1/x) least-squares linear regression analysis of the peak ratio of Doxorubicin to IS (Daunorubicin) versus the ratio of the amount of Doxorubicin to IS in each standard solution.

3.16.1 Sample processing

Serum and perfusate samples

Sample extraction and preparation for HPLC analysis were done under reduced light conditions. Frozen samples (kept at -80°C) were thawed and mixed by vortex. Into a set of 1.5 mL Eppendorf tubes, 10 µL perfusate or 40 µL serum samples were added and followed by addition of 20 µL of a Daunorubicin solution (containing 200 ng) prior to the protein precipitation step. The tubes were briefly mixed by vortex and 600 µL of acetonitrile were added before another mixing by vortex (10 minutes at room temperature). Samples were centrifuged at 20000 x g for 10 minutes at 4°C. The supernatants were carefully removed with Pasteur pipets and transferred into a set of conical tubes. 600 µL of acetonitrile were added to the tubes containing pellets followed
by another mixing by vortex (5 minutes at room temperature). Samples were centrifuged at 20000 x g for 10 minutes at 4° C. The supernatants were carefully removed with Pasteur pipets and combined with the previous extracts into conical tubes. Combined extracts were evaporated to dryness at 35°C under a stream of nitrogen. The dried residues were solubilized in 200 µL HPLC buffer (acetonitrile/1-heptanesulfonic acid (0.2%) pH 4.0, 15/85). After mixing by vortex and a short centrifugation, samples were transferred into 200 µL micro inserts, placed into 1.5 mL amber (i.e. protected from light) autosampler vials and caped.

**Lung tissues sample processing**

Sample extraction and preparation for HPLC analysis were done under reduced light conditions. Frozen samples (kept at -80°C) were kept frozen in dry ice until they were cut and homogenized in 2 mM KH2PO4 pH 3.8 buffer (5 mg/ 0.5 mL) using a Polytron PT1200E for 15 seconds on ice. After homogenization, 500 µL of tissue homogenates (corresponding to 5 mg tissues) were transferred into 1.5 mL Eppendorf tubes followed by addition of 20 µL of a Daunorubicin solution (containing 200 ng). The tubes were mixed by vortex (5 minutes) prior to the protein precipitation step. A volume of 300µL acetone and 50 µL of aqueous zinc sulfate (70% solution) were then added to sample homogenates that were mixed by vortex (10 minutes at room temperature). Samples were centrifuged at 20000 x g for 10 minutes at 4° C. The supernatants were carefully removed with Pasteur pipets and transferred into a set of conical tubes. 300 µL of acetone were added to the tubes containing pellets followed by another mixing by vortex (5 minutes at room temperature). Samples were centrifuged at 20000 x g for 10 minutes at 4°C. The
supernatants were carefully removed with Pasteur pipets and combined with the previous extracts into conical tubes. Combined extracts were evaporated to dryness at 65°C under a stream of nitrogen. The dried residues were solubilized in 200 µL HPLC buffer (acetonitrile/1-heptanesulfonic acid (0.2%) pH 4.0, 15/85). After mixing by vortex and a short centrifugation, samples were transferred into 200 µL micro inserts, placed into 1.5 mL amber (i.e. protected from light) autosampler vials and caped.

3.17 Ifosfamide analysis in samples

Ifosfamide was purchased from Pharmaceutical Partners of Canada (PPP), trofosfamide from Toronto Research Chemicals, and Daunorubicin, 1-heptanesulfonic acid, potassium phosphate (KH2PO4) were purchased from Sigma. Acetonitrile of HPLC grade, acetone, hydrochloric acid (HCl) and acetic acid (glacial) were obtained from Caledon (Canada). Purified water was obtained from a milli-Q Advantage A10 apparatus (Millipore, Bedford, USA).

For serum and perfusate samples, HPLC was the used technique. The liquid chromatography system was the same as aforementioned before, only added an Ultimate 3000 Photodiode Array detector (Dionex) set at 195 nm. The chromatographic separation, the delivery of the mobile phase and the chromatographic data acquisition were the same used for the analysis of Doxorubicin samples. The column was initially equilibrated with mobile phase (acetonitrile/1-heptanesulfonic acid (0.2%) pH 4.0) 15/85
to get a stable baseline followed by 2 injections of a standard mixture containing Ifosfamide and Daunorubicin (used as internal standard).

For each set of experiment (serum and perfusate samples), an Ifosfamide standard curve (spiked with Daunorubicin: 200 ng) was generated from 0 to 200 µg in 100µL final volume. Quantitative analysis of Ifosfamide was performed by using the IS method. Calibration curves were obtained by linear weighted (1/x) least-squares linear regression analysis of the peak ratio of Ifosfamide to IS (Daunorubicin) versus the ratio of the amount of Ifosfamide to IS in each standard solution.

For lung tissue samples, Liquid Chromatography/Mass Spectometry (LC/MS) was used due to the highest sensitivity (0.1-0.2 ng) in comparison to HPLC (0.1-0.2 µg) for this particular drug. LC/MS was performed on a 1200 HPLC System (Agilent Technologies: Santa Clara, California, USA) and an API4000 Mass Spectrometer (ABSciex: Framingham, Massachusetts, USA). Chromatography ran at a flow rate of 500 µL/min on a Kinetex HILIC column 4.6x50mm, 2.6 µm (Agilent) isocratically at 10% A (90/10 Water/Acetonitrile 5 mM ammonium Formate pH 3.2) and 90% B (10/90 Water/Acetonitrile 5 mM ammonium Formate pH 3.2) over 3 minutes.

The mass spectrometer was operated in +ve APCI mode with a source temperature of 600°C. Precursor to product ion mass transitions was established using standard infusions. Data was acquired by MRM (Multiple Reaction Monitoring) with mass transitions monitored being 261.0→153.9 m/z for Ifosfamide and 323.1→153.9 m/z for Trofosfamide.
Quantitative analysis was carried out using Analyst 1.5.2 Software (ABSciex). Area ratios of integrated chromatographic peaks (analyte to trofosfamide used as internal standard) of unknown samples were plotted against standard curves generated using known mixtures of individual analytes and internal standard in order to calculate quantitative values.

3.17.1 Sample processing

Serum and perfusate samples

Sample extraction and preparation for HPLC analysis were done under reduced light conditions. Frozen samples (kept at -80°C) were thawed and mixed by vortex. Into a set of 1.8 mL Eppendorf tubes, 100 µL perfusate or 500 µL serum samples were added and followed by addition of 20 µL of a Daunorubicin solution (containing 200 ng) prior to the protein precipitation step. The tubes were briefly mixed by vortex and 1 mL of acetonitrile was added before another mixing by vortex (10 minutes at room temperature). Samples were centrifuged at 20000 x g for 10 minutes at 4°C. The supernatants were carefully removed with Pasteur pipets and transferred into a set of conical tubes. 1 mL of acetonitrile was added to the tubes containing pellets followed by another mixing by vortex (5 minutes at room temperature). Samples were centrifuged at 20000 x g for 10 minutes at 4°C. The supernatants were carefully removed with Pasteur pipets and combined with the previous extracts into conical tubes. Combined extracts were evaporated to dryness at 35°C under a stream of nitrogen. The dried residues were solubilized in 200 µL HPLC buffer (acetonitrile/1-heptanesulfonic acid (0.2%) pH 4.0, 15/85). After mixing by vortex and a short centrifugation, samples were transferred into
200 µL micro inserts, placed into 1.5 mL amber (i.e. protected from light) autosampler vials and caped.

**Lung tissue sample processing**

Sample extraction and preparation for LC/MS analysis were done under reduced light conditions. Frozen samples (kept at -80°C) were kept frozen in dry ice until they were cut and homogenized in acetonitrile/water (70/30) (10 mg/mL) using a Polytron PT1200E for 15 seconds on ice. After homogenization, 200 µL of tissue homogenates (corresponding to 2 mg tissues) were transferred into 1.8 mL Eppendorf tubes followed by addition of 50 µL of a trofosfamide solution (containing 50 ng). A volume of 500µL acetonitrile was then added to sample homogenates that were mixed by vortex (10 minutes at room temperature). Samples were centrifuged at 20000 x g for 10 minutes at 4°C. The supernatants were carefully removed with Pasteur pipets and transferred into a set of conical tubes. 1 mL of acetonitrile was added to the tubes containing pellets followed by another mixing by vortex (5 minutes at room temperature). Samples were centrifuged at 20000 x g for 10 minutes at 4°C. The supernatants were carefully removed with Pasteur pipets and combined with the previous extracts into conical tubes. Combined extracts were evaporated to dryness at 35°C under a stream of nitrogen. The dried residues were solubilized in 200 µL of 90/10 Water/Acetonitrile 5 mM ammonium Formate pH 3.2 After mixing by vortex and a short centrifugation, samples were transferred into 200 µL micro inserts, placed into 1.5 mL amber (i.e. protected from light) autosampler vials and caped.
3.18 Tissue preparation for ELISA

Lung tissue frozen previously stored on -80°C freezer was homogenized into a powder using a chilled mortar and pestle (on dry ice). The finely ground frozen tissue sample was carefully scooped into a chilled 2ml micro centrifuge tube using a chilled spatula. The amount of 50mg of lung tissue was then inserted into a micro centrifuge tube and 1 mL of lysis buffer added. These tubes containing these mixtures were placed in the pre cooled TissueLyser II for 2 minutes at 20 Hr. We then disassemble the adapter set, rotate the rack of tubes, in order to make that the tubes which were nearest to the TissueLyser II were then outermost, and reassembled the adapter set. The TissueLyser II operated for another 2 min at 20 Hr. We then carefully pipet the lysates into new micro centrifuge tubes and centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was transferred to a new micro centrifuge tube and stored at -80 degrees Celsius until analysis.

Supernatants of lung tissues were assayed using the specific ELISA kit for porcine IL-6, IL-8, TNF-α, and IL-1β (R&D Systems, Minneapolis, MN). The optical density of each well was read of 450 and 570 nm according to the manufacturer’s instructions with an NM-600 micro plate reader (Dynatech Laboratories, Chantilly, VA). The final concentration was calculated by converting the OD readings against a standard curve.
3.19 **Tunel staining**

Apoptosis was assessed by in situ terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining (In Situ Cell Death Detection Kit, TMR red, Penzberg, Upper Bavaria, Germany) according to manufacturer’s instructions. 4', 6-diamidino-2-phenylindole (DAPI) was used as the fluorescent nuclear stain. The tissue sections were stained and examined with a fluorescent microscope - Axiovert 200M inverted - (Zeiss, Oberkochen, Germany) using the software Axiovision 4.8. The images were taken by CoolSnap HQ 12 bit CCD camera (Roper, Ottobrunn, Germany). The areas of alveolar tissue were found using a 100x magnification. Magnification changed to 200x and the presence of cells was measured according to DAPI staining. The TUNEL-stained images in red were found and taken randomly in five areas of each tissue section. Apoptosis was considered when TUNEL and DAPI stain were found overlapping in the same area. The apoptotic cells (TUNEL and DAPI-positive) represents an average of the five areas examined in each slide.

3.20 **Fluorescence**

Doxorubicin has an auto fluorescence characteristic, thus the identification of this signaling could be used as a way to assess the distribution of this drug in lung tissue[110,140]. Lung tissue biopsies were collected at the end of reperfusion from three different areas of the lung (upper, middle and lower). In each sample, a mixture
constituted by 50% of TissueTek optimum cutting temperature (OCT) compound and 50% of Phosphate buffered saline (PBS) was injected until the sample looked “inflated”. The sample was then mounted on OCT compound and put on dry ice. After the OCT block was frozen, it was wrapped in foil and store in an -80°C freezer.

Each OCT block was sectioned in 10 µm thick, mounted on glass slides, and allowed to air-dry. Doxorubicin auto fluorescence was detected using a Zeiss Axiovert 200M inverted microscope with a 100 W HBO mercury light source equipped with a 530 to 560 nm excitation and a 573 to 647 nm emission filter set. Tissue sections were imaged with a FLUAR 10/0.50 NA lens and captured with a Roper Scientific CoolSnap HQ CCD camera. Tissue sections were tiled using a motorized stage so that the distribution of Doxorubicin was obtained for the entire tissue section. All images were captured in 8-bit signal depth and subsequently pseudo-colored[140].

3.21 Statistical analysis

Results were analyzed using Graphpad Prism 5 (GraphPad Software Inc, La Jolla, Calif). Data is expressed as mean ± standard error of the mean. For comparisons in lung ventilatory and perfusion parameters, Repeated Measures Analysis of Variance (ANOVA) was utilized. Post-test analysis between each time point was performed with Tukey test for multiple comparisons. For comparisons between pathological injury scores, Friedman test was utilized, (post-test analysis was done with Dunn test). For comparisons in pharmacokinetics, Unpaired T-test was used when 2 groups were
compared and One-way ANOVA when 3 or more groups were analyzed (post-test analysis between each time point was performed with Tukey test for multiple comparisons). For inflammatory profile and apoptosis measurement, One-way ANOVA was utilized. P values less than 0.05 were considered significant.
4 Results
4.1 Phase I - Development of the large animal model

The translation of the EVLP to an IVLP model was performed using the same perfusate solution and modifications in the parameters of the ventilatory strategy and perfusion strategy previously employed. We demonstrated that the perfusion of a lung in situ for an extended perfusion time is possible, without additional injury. The physiologic assessment showed stable oxygenation levels (P/F ratio), PVR (PVR), and airway dynamics (peak airway pressure [Pawp] and dynamic compliance [Cdyn]) of the left lung during IVLP (Figure 14).

![Figure 14 - Left lung function during IVLP.](image)

(A) Left lung oxygenation (P/F ratio, mmHg), (B) PVR (PVR, dynes/sec/cm$^5$), (C) Peak airway pressure (Pawp, cmH$_2$O) and (D) Dynamic compliance (Cdyn, cmH$_2$O/ml) from beginning to four hours of IVLP (n=6; P>0.05 between measures of lung function in all time-points during IVLP, Repeated Measures ANOVA).
The physiologic assessment of the perfused lung was also analyzed in comparison to baseline and reperfusion levels. This is an important comparison considering that blood reperfusion may be a source of injury as well. Herein, we demonstrated that the stable physiologic assessment of the perfused lung (left) during prolonged IVLP was maintained during the 4h reperfusion period (Figure 15).

![Figure 15](image)

**Figure 15 - Left lung function during the entire procedure.** (A) Left lung oxygenation (P/F ratio, mmHg), (B) Peak airway pressure (Pawp, cmH₂O) and (C) Dynamic compliance (Cdyn, cmH₂O/ml) comparing before, during four hours of IVLP and additional four hours of reperfusion (n=6; P<0.0001 between P/F ratio in baseline and during IVLP time-points. P>0.05 between measurements in Pawp and Cdyn in all time-points during IVLP, Repeated Measures ANOVA).

We also demonstrated that the histology of the perfused lung was preserved in comparison to a baseline biopsy (performed at the beginning of the experiment) and to a
sample collected after 4h reperfusion, using the modified Ginsberg score to analyze signs of acute lung injury. This assessment was done in a blindly fashion by a medical pathologist experienced in acute lung injury (David Hwang). The result of this analysis is described in Figure 16.

Figure 16- Histologic assessment of the perfused lung. Biopsies compared before (A), after IVLP (B), and after reperfusion (C). Magnification 200x. The histologic pattern of the left lung was preserved at the 3 time-points. No significant differences were found between the three time-points (n=6; P>0.05 between measures in all time-points, Friedman test).

Chest X-Rays may also reveal signs of acute lung injury, like pulmonary infiltrates. In order to assess potential radiologic signs of lung injury, an image was acquired before the
chest was open and another image at the end of the entire experiment (IVLP + reperfusion). These images were compared in order to analyze potential differences that might be inducted by the procedure itself. During the development of the large animal model of IVLP, no significant differences were verified (Figure 17).

![Chest X-ray images](image)

**Figure 17 - Chest X-ray of an animal subjected to 4 hours of IVLP and 4 hours of reperfusion.** (A) Baseline X-ray, at the beginning of the procedure, before IVLP. (B) At the end of the IVLP plus reperfusion periods. The left lung, which was submitted to IVLP, remains with a preserved pattern when we compared (A) and (B).

One of the main gaps of previous studies was the heterogeneous distribution of chemotherapy in lung tissue. So, before we started the studies associating IVLP + chemotherapy, it was important to determine if our IVLP strategy would allow a good distribution of the chemotherapy. In anticipation to the studies with chemotherapy and in order to analyze the distribution of the perfusate in the lung, we performed experiments to analyze the distribution of the perfusate in the lung during long-term IVLP injecting
India Ink in the perfusate solution and we demonstrated that the stain was macroscopically well distributed in lung tissue after 4h of perfusion (Figure 18).

**Figure 18 - The distribution of India ink after IVLP.** (A) The whole left lung is harvested and sectioned transversally. Macroscopic visualization of the stain is demonstrated in the upper (B), middle (C) and lower (D) parts of the lung.

In summary, these results demonstrated that the IVLP showed stable physiologic function during all the steps of the experiment, without evidences of histologic acute lung injury neither radiologic. Additionally, an estimative of the distribution could be assessed with India ink, towards a good distribution in the lung.
4.2 Phase II - Studies with chemotherapy

The logical next step was to conduct studies using the IVLP strategy developed with chemotherapy. Doxorubicin was the drug of choice to start the studies considering the high efficacy of this drug for patients with metastatic sarcoma and the narrow therapeutic index of this drug systemically. The severe side effects of Doxorubicin, such as cardio toxicity, can be minimized with the IVLP platform, assuming that with this approach, very limited systemic exposure of the drug may happen.

In order to avoid technical problems with the double-lumen endotracheal tube used during the development of the large animal model of IVLP like obstructions and migrations, which could potentially influence the ventilation of the lung and, as a consequence, the perfusion of the organ in any specific area, we decided to use a single-lumen endotracheal tube, enabling an homogeneous ventilation of both lungs. Thus, the airway dynamics (peak airway pressure and dynamic compliance) measured in this part of the study reflects the function of both (perfused and non-perfused lungs). The P/F ratio and the PVR (measured only during IVLP) reflect the left lung function only.

Using the developed IVLP platform, we administered 75 mg/m$^2$ of Doxorubicin, calculated according to the body surface of the animal, to the perfusion circuit and we demonstrated that the same dose that is administered each 21 days in a clinical setting could be administered in bolus and perfused within a closed system for an extended perfusion time.
The P/F ratio showed excellent levels even after the administration of chemotherapy, and remained stable during reperfusion. The PVR remained stable when compared before and after the addition of chemotherapy to the perfusion circuit (Figure 19).

Figure 19 - Left lung function during IVLP with Doxorubicin 75 mg/m$^2$. (A) Left lung oxygenation (P/F ratio, mmHg), comparing before, during four hours of IVLP and additional four hours of reperfusion and (B) PVR (PVR, dynes/sec/cm$^{-5}$) of the left lung during IVLP, where baseline is the PVR immediately before the addition of chemotherapy to the perfusion circuit, followed by four hours of perfusion. (n=4; P<0.0001 between P/F ratio comparing baseline and 2, 3 and 4 hours of IVLP. P>0.05 between measurements in PVR in all time-points, Repeated Measures ANOVA).

The airway dynamics also remained stable during the procedure. In relation to the EVLP concept, these parameters were early indicators of lung injury. The increase in the peak airway pressure and the drop in the dynamic compliance, associated with an increment in the PVR, usually happened earlier than modifications in the oxygenation levels[108,142]. Hence, considering the similarities between IVLP and EVLP, a preserved pattern in the
airway dynamics is important. We observed stable airway pressures and lung compliance during IVLP and after blood reperfusion (Figure 20).

Figure 20 – Airway dynamics during IVLP with Doxorubicin 75 mg/m². During long term IVLP with chemotherapy, lung function remained stable. (A) Peak airway pressure (Pawp, cmH₂O), (B) Airway plateau pressure (Pplat, cmH₂O), (C) Dynamic compliance (Cdyn, cmH₂O/ml) and (D) Static compliance (Cstat, cmH₂O/ml) from baseline until four hours of reperfusion (n=4; P>0.05 between measures of lung function in all time-points during the entire procedure, Repeated Measures ANOVA).

When we analyzed the histology of the perfused lung with Doxorubicin 75 mg/m², we did not find statistical significance in the three time points analyzed. Interestingly, during the development of IVLP model, we found the lowest histologic injury scores in the sample collected at the end of the perfusion (Figure 16) and in this part of the study,
the injury score found in the sample collected at the same time point increased. Even with this increment in the injury, the histology pattern remained mostly preserved (Figure 21).

**Figure 21 - Histologic assessment of the perfused lung with Doxorubicin 75mg/m².** A comparison was done before (A), after IVLP (B), and after reperfusion (C). The histologic pattern of the left lung was preserved at the 3 time-points. We can see the analysis done by a pathologist (David Hwang) (D), showing that no significant differences were found between Pre IVLP (green), Post IVLP (red) and Post reperfusion (yellow). Magnification 200x. (n=4; P>0.05 between measures in all time-points, Friedman test).

Radiologic images were also recorded from these studies, and the perfused lung remained with a very similar radiological pattern in comparison to the images acquired at the beginning of the experiment, before the addition of chemotherapy to the perfusion circuit (Figure 22).
Figure 22 - Chest X-ray of an animal subjected to 4 hours of IVLP and 4 hours of reperfusion with Doxorubicin 75mg/m². (A) Baseline X-ray, at the beginning of the procedure, before IVLP. (B) At the end of the IVLP plus reperfusion periods. The left lung, which was submitted to IVLP, remains with a preserved pattern when we compared (A) and (B).

Thus, we showed that IVLP with Doxorubicin 75 mg/m² was safely administered using the developed IVLP strategy, based on physiologic data, histology samples and radiologic images.

The effects of the administration of two drugs using the IVLP strategy were also assessed. Although this approach was previously tested in small animals, no reports were available in a large animal model. In this setting, standard doses of Doxorubicin (75mg/m²) and Ifosfamide (6g/m²) were added to the perfusion circuit at the beginning of
IVLP. Even anticipating that additional toxicity could be expected using two drugs in combination, we found no additional lung toxicity compared to IVLP performed with single agent Doxorubicin at 75 mg/m². In addition to this, no increments in the PVR of the perfused lung were noticed when we compared before and after the addition of this multidrug regimen in the perfusion circuit. (Figure 23)

**Figure 23 – Left lung function during IVLP with two drugs.** (A) Left lung oxygenation (P/F ratio, mmHg) before, during four hours of IVLP and additional four hours of reperfusion and (B) PVR (PVR, dynes/sec/cm⁻⁵), assessed during IVLP before and after the addition of Doxorubicin + Ifosfamide (n=3; P=0.0003 between P/F ratio in baseline and during the third and fourth hours of IVLP. P>0.05 between measurements in PVR during IVLP, Repeated Measures ANOVA).

The analysis of the airway dynamics measured before IVLP, during IVLP with the combined drug regimen and during the 4 hours reperfusion period also followed the trend previously described for the IVLP without chemotherapy and with Doxorubicin 75 mg/m² (Figure 24).
Figure 24 – Airway dynamics during IVLP with two drugs. (A) Peak airway pressure (Pawp, cmH$_2$O), (B) Plateau pressure (Pplat, cmH$_2$O), (C) Dynamic compliance (Cdyn, cmH$_2$O/ml) and (D) Static compliance (Cstat, cmH$_2$O/ml) comparing before, during four hours of IVLP and additional four hours of reperfusion (n=3; P>0.05 between measures of lung function in all time-points, Repeated Measures ANOVA).

Additional analysis was done in histologic samples collected before, immediately after IVLP and at the end of additional four hours of blood reperfusion. We did not find significant signs of acute histologic injury in comparison of the three samples collected during the experiments performed before with a mixed scheme of drugs (Figure 25). These results demonstrated that Doxorubicin 75 mg/m$^2$ plus Ifosfamide 6 g/m$^2$ could be safely administered during IVLP.
Figure 25 - The histologic assessment of the lung perfused with two drugs. Comparisons done before (A), immediately after IVLP (B), and after reperfusion (C). The histologic pattern of the left lung was preserved at the 3 time-points. Magnification 200x. No significant differences were found between the three time-points (n=6; P>0.05 between measures in all time-points, Friedman test).

For a matter of comparison, previous clinical phase-1 studies established that the maximal tolerated dose of Doxorubicin was within a range of 40 and 60 mg/m². Hence, even considering that our project focused only acute lung injury, we demonstrated that a greater dose of Doxorubicin and even the combination of this drug with Ifosfamide was possible and safe.
In order to test the limits of our strategy and also to further assess the effects of IVLP with higher doses of Doxorubicin, IVLP with two (150mg/m\(^2\)) and three (225 mg/m\(^2\)) times the standard dose was performed.

Two animals were submitted to 4 hours IVLP with 150 mg/m\(^2\) of Doxorubicin and a fall in the oxygenation levels of the left lung during reperfusion was found, while the other parameters analyzed remained stable (Figure 26).

**Figure 26 - Lung function of the two animals submitted to IVLP with Doxorubicin 150 mg/m\(^2\).** Pig 1 (purple line) and pig 2 (green line). Left lung oxygenation (P/F ratio, mmHg), (B) PVR (PVR, dynes/sec/cm\(^{-5}\)), (C) Peak airway pressure (Pawp, cmH\(_2\)O) and (D) Dynamic compliance (CDyn, cmH\(_2\)O/ml) from beginning to four hours of IVLP (n=2).
The histology samples collected at the end of IVLP and reperfusion did not show severe signs of lung injury. The interesting finding here was that, despite the lung having normal appearance and function during IVLP, a major difference was found after reperfusion, where the lung had worsening oxygenation and with an edematous appearance (Figure 27).

Figure 27 – Histologic and macroscopic assessment of the lung perfused with Doxorubicin 150 mg/m². Lung histology at the end of IVLP (A) and reperfusion (B). Macroscopic view of the left lung at the end of IVLP (C) and at the end of reperfusion (D), where the lung appearance shows congestion and edema. These findings were present in the two pigs submitted to IVLP with Doxorubicin 150 mg/m². Magnification 200x in A and B.

To characterize dose related toxicity, we then studied the effect of the administration of three times the standard dose of Doxorubicin in two pigs submitted to IVLP and for this
purposed, 225 mg/m² of the drug was injected in the perfusion circuit. In relation to the parameters analyzed, we found a preserved P/F ratio, but increments in the PVR of the left lung were noticed after the addition of such a high level of chemotherapy to the circuit. The airway dynamics also showed that the Pawp increase and the Cdyn decreased over time during IVLP (Figure 28).

![Graphs showing P/F ratio, PVR, Pawp, and Cdyn](image)

**Figure 28 - Lung function of the two animals submitted to IVLP with Doxorubicin 225 mg/m².** Pig 1 (blue line) and Pig 2 (red line). Left lung oxygenation (P/F ratio, mmHg), (B) PVR (PVR, dynes/sec/cm⁻⁵), (C) Peak airway pressure (Pawp, cmH₂O) and (D) Dynamic compliance (CDyn, cmH₂O/ml) from beginning to three hours of IVLP (n=2).

The severe lung injury found during this part of the study was characterized by massive lung edema of the left lung and the presence of a large amount of liquid in the endotracheal tube, which was attributed as lung edema and the presence of perfusate edema.
solution (because the amount of perfusate dropped significantly at this part of IVLP).

These findings are highlighted in Figure 29.

![Figure 29](image)

**Figure 29 – Macroscopic signs of lung injury verified during IVLP with Doxorubicin 225 mg/m².** (A) Lung injury during IVLP, with severe edema of the left lung. (B) After euthanasia of the pig, a median sternotomy was performed and a striking difference could be noticed between the right (non-perfused) and the left lungs. (C) The endotracheal tube that was ventilating the pig was filled with liquid during IVLP, which is typically found in cases of severe lung edema.

As discussed previously, no data is available during reperfusion due to severe lung injury that lead to the early termination of both experiments. The two animals were euthanized at the end of the second and third hour of IVLP, due to massive lung edema. Histological assessment could only be performed during IVLP and edema in the intraalveolar septum was the main histologic feature (not reported here).
The inflammatory profile was assessed in lung tissue analyzing the levels of IL-6, IL-8, TNF alfa and IFN 1-β. The samples were collected before IVLP (pre perfusion) and at the end of reperfusion. Only IL-8 was found to have significant differences in the reperfusion samples in comparison to pre perfusion when IVLP with Doxorubicin 150 mg/m² was performed (Figure 30). We also recognize the limitations of this analysis considering that two animals were studied at this level of Doxorubicin.

**Figure 30 – Inflammatory profile in lung tissue.** Inflammatory cytokines were measured in lung tissue before and at the end of reperfusion. Highlighted in blue are the samples collected pre perfusion (before IVLP) and after 4h IVLP + 4h reperfusion the following groups were analyzed: without chemotherapy (green), with Doxorubicin 75 mg/m² (purple), with Doxorubicin 75 mg/m² + Ifosfamide 6g/m² (red) and with Doxorubicin 150 mg/m² (yellow). In the group where IVLP was performed with Doxorubicin 150 mg/m², there was a significant increase in the IL-8 levels in lung tissue in comparison to pre perfusion samples (A). Changes in IL-8 levels related to the other groups and the levels of (B) IL-6, (C) IL 1- ß and (D) TNF alfa were not statistically significant. (P=0.02 between pre perfusion and Doxorubicin 150 mg/m² IL-8 levels. P>0.05 between measures of cytokines levels in all time-points, One-way ANOVA).
Doxorubicin is a drug that can lead to apoptosis[152], and considering the high levels that were administered exclusively to the left lung, apoptotic cells might be found in lung tissue. In models of ischemia-reperfusion injury, apoptosis can usually be found during reperfusion may lead to programmed cell-death, thus we analyzed samples collected at the end of reperfusion using TUNEL staining[153]. We found more apoptotic cells in the cases submitted to IVLP with 150 mg/m² of Doxorubicin, which points to the fact that drug-induced injury might be the cause of these findings (Figure 31). No assessment was possible in the animals perfused with 225 mg/m² of Doxorubicin because euthanasia was required during IVLP.

**Figure 31 - Apoptosis Imaging.** Tunel staining (red spots) was performed to identify apoptotic cells in samples collected after 4 hours of IVLP plus 4 hours of reperfusion and is described according to each group. (A) IVLP without chemotherapy (B) IVLP with Doxorubicin 75 mg/m² (C) IVLP with Doxorubicin 75 mg/m² + Ifosfamide 6 g/m² and (D) IVLP with Doxorubicin 150 mg/m². (E) Quantification of Tunel positive cells was assessed, and despite the fact that statistical significance was not found, a trend to a large number of apoptotic cells can be identified in the IVLP with 150 mg/m² of Doxorubicin group.
The analysis of the pharmacokinetics of the drugs during the experiments allowed us to find the levels of Doxorubicin in perfusate, serum and lung tissue, using HPLC.

The perfusate levels of Doxorubicin peaked at one hour of IVLP, with following descendant values. No chemotherapy was found in systemic circulation in serum samples collected at the end of IVLP and at the second hour of reperfusion. It is very important to highlight that an efficient separation between the IVLP circuit and the systemic circulation was identified. Additionally, we found a good distribution of Doxorubicin in lung tissue, due to the presence of equivalent levels of Doxorubicin in the three different areas of the lung analyzed at the end of reperfusion (Figure 32).

Figure 32 – Tissue and perfusate levels measured in experiments with Doxorubicin 75 mg/m². (A) Tissue levels of Doxorubicin were measured with HPLC, and analyzed in three different areas of the lung – upper, middle, lower - at the end of reperfusion. Per fusate levels were also assessed with HPLC 10 minutes after administration of chemotherapy in the perfusion circuit, followed by hourly assessments. Serum levels of Doxorubicin were negligible (n=8; P>0.05 between measures of tissue levels of Doxorubicin at the end of reperfusion in all time-points, One-way ANOVA).
Regarding Ifosfamide pharmacokinetics, the distribution in lung tissue followed the same pattern of Doxorubicin and the perfusate levels peaked at the second hour of IVLP (Figure 33).

The concentration of Ifosfamide in lung tissue was analyzed with mass spectrometry due to the low sensitivity of HPLC in this setting. The perfusate levels of Ifosfamide were assessed with HPLC and small levels of this drug were found in the systemic circulation, due to the highest sensitivity of this method.

**Figure 33 – Pharmacokinetics of Ifosfamide.** (A) Tissue levels of Ifosfamide were measured with Mass Spectrometry, and analyzed in three different areas of the lung – upper, middle, lower - at the end of reperfusion. Perfusate levels were assessed with HPLC immediately after administration of chemotherapy in the perfusion circuit - 10 min – and followed by hourly assessments. Serum levels of Ifosfamide were assessed at the end of IVLP and at the second hour of reperfusion and small levels were found (not presented graphically) due to the high sensitivity that HPLC has to identify levels of this drug in comparing to Doxorubicin. (n=3; P>0.05 between measures of tissue levels of Ifosfamide at the end of reperfusion in all time-points, One-way ANOVA)
We again found that an effective separation between the systemic circulation was possible. Moreover, a homogeneous distribution of the drugs was found in lung tissue at the end of reperfusion.

Considering the cases where significant toxicity was found, the perfusate levels of the pigs submitted to IVLP with 225 mg/m² and 150 mg/m² of Doxorubicin were much higher than those observed at the level where IVLP was considered safe (75 mg/m²). In fact, assuming the patterns of injury described, these perfusate levels of circulating Doxorubicin may be a potential explanation for the lung injury verified in both groups.

Figure 34 – Comparison in the perfusate and tissue levels of Doxorubicin obtained in the dose-escalating IVLP experiments. (A) Perfusate levels Doxorubicin. Higher levels were found in the 225 group (n=2), compared to the 150 (n=2) and 75 mg/m² (n=8). (B) Tissue levels analyzed in three areas of the lung – upper, middle, lower - at the end of reperfusion and compared to the group submitted to IVLP with 150 (n=2) and 75 mg/m² (n=8). P>0.05 between measures of tissue levels of Doxorubicin at the end of reperfusion in all areas of the perfused lung, One-way ANOVA.
The tissue levels of Doxorubicin assessed at the end of reperfusion in the 150mg/m² group were not statistically different in comparison to the 75mg/m² group, although a clear trend towards higher tissue levels in the higher administrated dose was observed (Figure 34).

We also analyzed the influence that the perfusion time might have in the final tissue levels of Doxorubicin. For this purpose, pigs were submitted to IVLP for 2 or 4 hours, followed by 4 hours of reperfusion. Significantly higher tissue levels of chemotherapy were found in the latter group, pointing to the fact that an extended perfusion time may allow higher final tissue levels of Doxorubicin (Figure 35).

**Figure 35 – Influence of duration of IVLP in the final concentration of Doxorubicin in lung tissue.**

Pigs submitted to different periods of IVLP followed by 4 h of reperfusion. Tissue levels of Doxorubicin assessed after 2h IVLP + 4h reperfusion (n=5, yellow) and 4h IVLP + 4h reperfusion (n=8, green). (P=0.003 between measures in the two groups, unpaired T-test.)
The identification of the auto fluorescence of Doxorubicin allowed us to analyze the distribution of this drug in lung tissue. A negative control was used (pig lung perfused with Steen solution only) and, in the IVLP cases with Doxorubicin 75 mg/m² we could observe a strong signal of the fluorescence (highlighted in red) distributed in three different areas of the lung (Figure 36)

Figure 36 – Auto fluorescence of Doxorubicin. The identification of the auto fluorescence of Doxorubicin (red) in samples mounted on OCT blocks. (A) Negative control – healthy pig lung submitted to 6 hours EVLP with Steen solution only. (B) Upper area of a healthy pig lung submitted to IVLP with Doxorubicin 75mg/m² lung, and the same lung was also assessed in the (C) Middle and (D) Lower areas.

This auto fluorescence will be particularly important for future efficacy studies, where the presence of Doxorubicin may be verified and even quantified in experimental animal models of induced-lung metastases.
We also found an interesting correlation between bronchial circulation and tissue levels of chemotherapy. Reviewing the first thirteen IVLP cases, we found that some specific experiments demonstrated lower levels of chemotherapy in lung tissue compared to others (around 10 times less). Assessing the reports of each surgical procedure, we found that the pigs where final lung tissue levels of chemotherapy were much smaller had in their records descriptions like “hemodynamic instability” and “increments in the levels of the perfusate in the reservoir of the circuit, with a bloody aspect of the perfusate solution”. This lead us to hypothesize that the bronchial circulation might be related to these findings and despite the fact that we tried to control the bronchial circulation by stripping the tissue around the left main bronchus since the beginning, we found that in some cases, probably this “control” was not optimal. In fact, a suboptimal control of this circulation in association with an extended perfusion time was leading to hemodynamic instability and presumably to the dilution of chemotherapy that was circulating within the circuit.

Thus, we decided to analyze the levels of hemoglobin in the perfusate at the end of IVLP (assuming that cases with suboptimal control of the bronchial circulation would present with higher levels of hemoglobin in the perfusate compared to cases where the bronchial circulation was effectively controlled), and we found a strong inverse correlation between with higher levels of hemoglobin and lower tissue levels of Doxorubicin (Figure 37).

After we suspected from this association and confirmed it, a lot of attention was paid to the proper dissection and isolation of the bronchial circulation around the left main bronchus. Interestingly, these measures solved the problem, because no more issues
were verified, either considering the hemodynamic status of the animal during IVLP and of the final tissue levels of Doxorubicin in lung tissue. Thus, bronchial circulation seems to be an important issue in long-term IVLP and needs to be properly addressed.

**Figure 37 – Influence of bronchial circulation in the final tissue levels of Doxorubicin.** (A) The two vials on the left are perfusate samples that were collected at the end of IVLP from pigs with elevated final lung levels of Doxorubicin and low levels of hemoglobin, whereas the two on the right were from experiments where lower levels of chemotherapy and high levels of hemoglobin in perfusate were found. (B) An inverse correlation was found between the hemoglobin (g/L) and chemotherapy levels (ng/mg of lung tissue). (Pearson correlation between the two variables analyzed $r^2 = -0.8481$ and $p=0.0002$).

We also analyzed the pharmacokinetics of Doxorubicin using a lung-free perfusion circuit, in order to study if the observed drop in the perfusate levels that happened over time during the IVLP experiments as highlighted in the Figure 32-B was not due to the fact that the chemotherapeutic drugs might being bounded to the plastic tubing, leukocyte filter, membrane oxygenator and other elements of the circuit. If this was happening, it could lead to misleading results, because our assumption was that the chemotherapeutic
drug was actually being absorbed by the lung tissue over time and not binded to the perfusion circuit and its components. The analysis done using this lung-free circuit allowed us to find that the perfusate levels of chemotherapy were not reducing over time (Figure 38), pointing to the fact that the drug was not being binded to the perfusion circuit during IVLP.

![Perfusate levels - (n=3)](image)

**Figure 38 – Pharmacokinetics of chemotherapy in a lung-free circuit.** Doxorubicin 75 mg/m$^2$ injected in a closed perfusion circuit and perfusate levels analyzed over time. In all three cases performed, the levels of chemotherapy measured in the perfusate do not drop over time.

### 4.3 Phase III – Human lungs

A pre-clinical model using human lungs that were rejected for clinical transplantation is an important tool to analyze the effects of the lung perfusion with chemotherapy in
human cells. In this experiment, we were also able to analyze the physiology of human lungs submitted to EVLP with high-dose chemotherapy.

We did three perfusions using, respectively, 75 mg/m$^2$, 150 mg/m$^2$ and 225 mg/m$^2$ of Doxorubicin (Figure 38).

Figure 39 – Human lungs perfused Ex Vivo with chemotherapy. (A) Lung perfused with 75 mg/m$^2$ of Doxorubicin for four hours (B) Lung perfused with 150 mg/m$^2$ of Doxorubicin (C) Lung perfused with 225 mg/m$^2$ of Doxorubicin.
The physiology of these three lungs was assessed during IVLP, before and after the addition of chemotherapy to the perfusion circuit. The lung number 2 (Figure 39-case2), which had the worst function at the harvest, in fact showed signs of lung injury typically found during IVLP (increase in the Pawp and decrease in the Cdyn, despite preserved oxygenation levels). The lung number 1 (Figure 39-case 1) showed stable function during EVLP with chemotherapy and, interestingly, the lung number 3 (Figure 39-case 3) also showed stable function during EVLP, even though a higher dose of chemotherapy was administered in comparison to lung number 2.

Figure 40 - Function of the human lungs submitted to EVLP with Doxorubicin. In this setting, Doxorubicin was administered at the following doses: 75 mg/m² (case 1), 150 mg/m² (case 2) and 225 mg/m² (case 3). Left lung oxygenation (P/F ratio, mmHg), (B) PA pressure (PAP, mmHg), (C) Peak airway pressure (Pawp, cmH₂O) and (D) Dynamic compliance (Cdyn, cmH₂O/ml) from baseline (before addition of chemotherapy) to four hours of IVLP.

113
Despite the fact that only one lung was perfused per group, we noticed that a lung with an baseline impaired function (Case 2) was not able to tolerate the effects of the chemotherapy in the perfusion circuit. Interestingly, the human lungs with baseline preserved function (Case 1 and Case 3) demonstrated stable lung parameters during the entire study, even when the dose of Doxorubicin administered was the same amount (225 mg/m²) that showed severe acute lung injury and massive lung edema during IVLP in the experiments performed in pigs.
5 Discussion
We described here a successful strategy of IVLP without inflicting lung injury for an extended period of time. Our strategy allowed the perfusion of a lung within a closed system without perfusion-related injury, and the addition of standard doses of chemotherapy to the system did not add significant acute injury to the lung.

This approach can be potentially used to diminish the chances of local recurrence after lung metastasectomy by administering high-dose chemotherapy or targeted therapeutic agents to the lung without the harmful systemic side effects.

In the past two decades, IVLP has been studied and the experimental and clinical data available so far demonstrated the feasibility and reproducibility of this modality as a potential treatment for pulmonary metastases[103,124]. Higher tissue concentrations were found with this approach when compared with systemic administration of chemotherapy[66], and further experiments showed that IVLP was effective in the treatment of lung metastases in small animal models [154,155]. However, despite the initial optimism that was generated with these experiments, the subsequent clinical studies using short-term evaluating different drugs (Doxorubicin, Cisplatin and Melphalan) and perfusate solutions (saline, colloids, dextran and blood) demonstrated important concerns related to acute lung toxicity and no improvement in overall survival[127,128,156], hindering the broad application of this technique. Importantly, most of these studies did not discriminate whether the toxicity was mainly related to the drug, perfusion solution, or actual technique of IVLP.

The IVLP approach has been recently employed with encouraging results in a clinical setting. A phase-1 clinical study using short-term (30 minutes) IVLP with Melphalan for
resectable lung metastases from tumors such as colorectal, kidney and sarcomas has been performed. Despite the fact that lung function was impaired at 1 month of follow-up, no major long-term toxicity was found.\cite{130} It is important to note that lung metastasectomy with IVLP can be performed with comparable results to conventional lung metastasectomy with respect to post-operative quality of life scores\cite{131}. Hence, an IVLP strategy that allows an extended perfusion time, minimize lung injury and enables the administration of higher drug doses and combined agents may help to improve outcomes.

Initially, our study aimed to demonstrate that no measurable acute lung injury occurs with our new IVLP strategy. The development of the large animal model of IVLP based on strategies that were modified from existing EVLP projects allowed us to perfuse a lung in situ for an extended time, without acute lung injury. This was important as the perfusion-ventilatory strategy previously used may have been a source of injury. The chemotherapeutic drug was generally appointed as the main (and in many studies, as the only) cause of lung injury but it is important to note that the perfusion and ventilatory strategy employed could contribute to this process.

Previous large animal studies have raised concerns about the potential injury that the perfusion system may inflict apart from the chemotherapeutic agent. Furrer\cite{110,157} performed IVLP for 15 minutes in pigs, using flow rates that ranged from 70-120 ml/min and found that controls animals (IVLP without chemotherapy) had the same pattern of acute lung injury (as examined by histology and chest x-ray) compared to animals perfused with Doxorubicin. In another study, pigs submitted to IVLP with and without
chemotherapy demonstrated higher histologic injury scores and impairment of functional parameters compared to pigs in a control sham group[108]. Histologic signs of hemorrhagic edema were found in both the control and Melphalan treated groups[103] highlighting the potential role of perfusion circuit injury. More recently, Pages[104] performed IVLP for 30 minutes in 50 kg pigs, with a perfusion flow ranging from 500-600 ml/min (adjusted to a PA pressure around 25 mmHg) and found that the control group had greater histological injury compared to lungs perfused with Gemcitabine reinforcing the possibility of injury resulting from IVLP itself.

With the objective to optimize previously reported IVLP techniques, we derived many technical aspects related to the perfusion and ventilatory strategies from our previously established EVLP technique used to preserve lungs for extended time periods for use in transplantation[158]. The EVLP technique has already proven clinical efficacy.[146]

The main aspects our strategy aspects of our modified IVLP technique included:

1) A perfusate solution with optimal osmotic pressure designed specifically for lung perfusion. This is a solution with high dextran content and albumin, leading to an optimal osmolality and oncotic pressure, while preventing the development of extravasation of intravascular fluid to the interstitial and alveolar space. This solution has been able to clear pulmonary edema in injured donor lungs. Previous and current used solutions for IVLP (saline solution, colloid solutions, blood) have already demonstrated to induce pulmonary edema in different animal models of lung perfusion[156,159]. Hence, a protective perfusate solution plays a key role in
2) A perfusion flow resulting in a reduction of hydrostatic pressure, thereby diminishing the chance of lung edema. In accordance with our previous studies with EVLP, here we perfused the left lung with 16% of CO. This rendered a PA pressure of 10-15 mmHg, which is much lower than other described IVLP techniques where in the majority of cases the flow is increased until PA pressure reaches 20 or 25 mmHg. By maintaining a lower hydrostatic pressure, the pulmonary vasculature is protected. Steen and colleagues have used PA pressure of 20 mmHg for EVLP and they have recognized that after 1h of EVLP, lungs begin to acquire pulmonary edema[138]. In contrast, our studies have shown no edema development up to 12h of perfusion[143]. Despite this low-pressure strategy, we did achieve homogenous distribution of perfusion over time. The linear relationship between perfusion flows, PA pressure levels and the development of pulmonary edema is well known[157]; thus, our flow rates, between 450-550 ml/min under PA pressure levels of 10-15 mmHg, may play an important role in lung protection.

3) A positive and physiological LA pressure preventing a collapse and reopening phenomenon, and protecting vascular endothelium. Although previous descriptions of IVLP models had controlled PA pressures, they did not exert any control over the drainage pressure in the pulmonary veins (drainage pressure). The described system here drains the effluent to a hard shell reservoir where the drainage pressure can be controlled and kept at physiological levels of 3-5 mmHg. We believe this is a crucial element for lung protection. A number of physiological experiments have been performed demonstrating that either negative pressures or supra-physiological LA pressures induce pulmonary edema and vascular failure[160,161].
4) Protective mechanical ventilation with lower tidal volumes, which prevent increases in alveolar perivascular pressure that can lead to increase in PVR and lung injury[162].

5) A mean PA pressure within close range to physiologic levels (10-15 mmHg). Increases in the PA pressure levels may correlate with acute lung injury in isolated lung perfusion[101]. Factors in addition to pump flow may also influence the PA pressure levels. These include a low pH in the perfusate, the presence of high tidal volumes, an impaired left atrial drainage, and technical problems with the catheters measuring these pressures. All of these factors must be corrected in order to have a stable and reliable PA pressure throughout the procedure.

6) The use of a centrifugal pump with better protection of lung vasculature. During the ventilator cycle, distension of the alveoli potentially can lead to increments in the pressure in the peri-alveolar vessels, resulting in increments in the PVR with cycle of breath. According to the way that the centrifugal pump works, increase afterload of the pump will result in decreased rotation and flow. The pump will not continue to push flow against higher resistance, avoiding potential injury.

7) The use of a membrane gas exchanger supplied with a special mixture of gas, which maintains the physiologic levels of CO₂ in the perfusate. This has been shown to be an important component of lung protection. This control seems to be an important step for the maintenance of a prolonged perfusion. These levels, whenever not within the proper range, may lead to endothelial injury and impairment of alveolar fluid re-absorption[147].
During the development of the IVLP large animal model, certain important intraoperative technical factors were identified:

1. **Proper position of vascular clamps** – Considering the small dimensions of the cannulas used to cannulate the left PA and the pulmonary veins, a clamp that is not in the proper position may generate obstruction or malfunctioning of these cannulas, leading to high levels of PA and LA pressures.

2. **Adequate drainage of the left pulmonary veins** – It is very important to dissect and properly isolate the left pulmonary veins. The dissection should be carried towards the LA, a proper site to anchor the purse string sutures. A well-placed cannula (as close as possible to the junction of the atrium and the respective vein) is the best way to avoid obstruction of the outflow drainage, allowing optimal drainage pressures.

3. **Bronchial circulation** – Systemic flow is drained to the reservoir during extended periods of IVLP and this may lead to instability of the hemodynamic status of the animal. To overcome this problem, a meticulous dissection was performed around the left main bronchus, including the removal on lymph nodes in this area and also the ligation of the bronchial vessels identified at this level.

The first step of this project consisted in develop a platform to perfuse a lung in situ without inflict acute lung injury. We were able to successfully modify the concepts developed for the EVLP platform, paying attention to specific issues encountered during the initial experiments and overcoming these difficulties. This protective IVLP platform developed was, then, used for experiments with chemotherapy as the logical next step of this project.
The chemotherapy studies performed in this project were based on the concept already explored in previous clinical IVLP studies: to administer to the perfusion circuit the dose of chemotherapy equivalent to the amount that is systemically administered in a clinical setting. This represents a large amount of chemotherapy being delivered to a target-organ.

Previous clinical IVLP studies[126,127] established Doxorubicin 40 mg/m$^2$ or 60 mg/m$^2$ as the maximal tolerated dose, with severe lung toxicity described at higher doses. Considering that the conventional dose administered for patients with metastatic sarcomas who are in a good performance status is Doxorubicin 75 mg/m$^2$ [150] and taking advantage of our IVLP strategy, we began the initial perfusions with this level of Doxorubicin, hypothesizing the our IVLP model could offer a protective environment for the lung. In fact, with this level of chemotherapy administered, we found only minimal and non-significant evidences of acute lung injury, as highlighted in the physiologic, histologic, radiologic and inflammatory profiles.

After these initial studies with Doxorubicin 75 mg/m$^2$, we then moved to IVLP with the combination of Doxorubicin plus Ifosfamide. Once again we demonstrated that no acute lung injury was found. Moreover, the association of two drugs was never reported in large animal or clinical IVLP studies, and we think that this warrants further exploration in a future phase-1 clinical study. The administration of doses beyond the previous maximal tolerated doses described clinically (Doxorubicin 60 mg/m$^2$) and the combinatory treatment of Doxorubicin 75 mg/m$^2$ plus Ifosfamide 6 g/m$^2$, safely
administered using our platform, could be a major progress towards a more effective treatment for patients with metastatic sarcomas to the lungs.

Dose-limiting studies were performed and the toxic levels studied were associated with two different patterns of acute lung injury. Reperfusion-injury was associated with two-times the standard dose of Doxorubicin (150 mg/m²). However, a careful assessment of these two animals showed that the P/F ratio had a slightly different pattern in comparison to the previous groups, presenting with a small drop after the second hour of IVLP, This suggests that the injury actually might have started during IVLP. We further extrapolate the dose of 150 mg/m², administering 225 mg/m² of Doxorubicin. Interestingly, a more severe pattern of acute lung injury was verified, with massive lung edema during IVLP, which lead to early termination of the experiments during IVLP. Taken together, we found that Doxorubicin 75 mg/m² alone or combined with Ifosfamide 6g/m² were the doses found to be safe in our study.

We then assessed the pharmacokinetics of Doxorubicin and Ifosfamide levels. For a direct comparison to previous experiments, only Doxorubicin was used because, to our best knowledge, there are no studies where Ifosfamide was used in IVLP. In contrast, Doxorubicin is a drug previously employed in several experimental and clinical IVLP studies.

The analysis of perfusate and tissue levels that we found in our study are discussed below:

**Perfusate:** in the groups perfused with Doxorubicin 75 mg/m², we found peak levels of 23 µg/ml after one hour of perfusion, with subsequent descendant values. In comparison
to previous large animal studies of IVLP where Doxorubicin was used, perfusate levels of 11 µg/ml [98] and 7 µg/ml [99] were associated with acute lung injury. Of note, we found lung toxicity with perfusate levels of 61.9 µg/ml, demonstrating that with our protective platform, higher levels of Doxorubicin are required to induce severe lung injury. An interesting comparison can be done with the pharmacokinetics of Doxorubicin after the administration of 50-90 mg/m² in clinical practice, where of plasma levels ranged from 0.8-1 µg/ml after 30 minutes of administration and 0.5-0.6 µg/ml after one hour of administration[69]. When compared to the levels found in our experiments, a significant increase in the levels of Doxorubicin was found at one hour of perfusion. Importantly, during our experiments, Doxorubicin was not detected in plasma during IVLP using HPLC (limit of detection of Doxorubicin with HPLC is 02 ng) indicating that our IVLP achieved an effective separation between pulmonary and systemic circulation.

**Tissue:** In the group where IVLP was performed with 75 mg/m², we found mean tissue levels of 61.82 µg/g at the end of 4 hours of IVLP plus 4 hours of reperfusion. Previously, Johnston et al. demonstrated that irreversible lung damage was found with Doxorubicin levels of 10-20 µg/g tissue [96]. Minchin et al. found vascular injury and alveolar epithelial necrosis when the levels of Doxorubicin were around 28 µg/g in lung tissue[98]. In another study, tissue levels higher than 20 µg/g were associated with lung injury (moderate pulmonary edema, interstitial infiltration and alveolar disruption)[99]. Severe signs like hemorrhagic lung edema were found at tissue levels of Doxorubicin of 21.9 µg/g of lung tissue[110]. It is important to stress that the tissue levels found in the group that we considered safe were 2-3 times higher than the previous described levels associated with lung injury. Furthermore, in our study, lung injury was found at tissue...
levels of 120 µg/g of Doxorubicin, which is 6-8 times higher than previously reported levels.

Even considering that our experiments were performed in pigs, a comparison can be done with the clinical IVLP studies in the past using Doxorubicin where the dose was calculated according to the body surface. In our study, the tissue levels found in the group where the IVLP was safely performed were close to 10x higher than the group that received the maximal tolerated dose of Doxorubicin[124,126], suggesting that a protective IVLP strategy may be associated with less lung injury even at much higher tissue levels of drugs.

We found that Doxorubicin was well distributed in the three areas of the lung analyzed at the end of reperfusion. This is an important concept considering the oncological purpose of this research. Whereas previous studies demonstrated that short-term IVLP was associated with an heterogeneous distribution of the drug in lung tissue[120], the fact that we could identify equivalent levels of Doxorubicin in many sites of the lung is an important finding. Considering that we aim to treat an organ with micrometastastic disease, ideally chemotherapy must reach the whole lung to be more effective. The higher perfusion flows that were possible using our protective platform may also contributed to a better distribution of drugs.

A unique characteristic of this project is the fact that we were able to perfuse a lung in a safe way for an extended period of time with higher doses of Doxorubicin. This was only possible because we demonstrated here that an optimized IVLP strategy is a key part for the successful IVLP strategy.
In fact, we showed that IVLP could be performed for 5-12 times longer time than previous experimental and clinical reports. The only study that tried to maintain IVLP for four hours demonstrated lung injury and impairment of function of the perfused lung at the end of IVLP, with a slight improvement after one hour of blood reperfusion[100]. The Table 6 highlights the key aspects found in our research in comparison to previous large animal studies where IVLP with Doxorubicin was performed.

<table>
<thead>
<tr>
<th>Author/year</th>
<th>Perfusion time</th>
<th>Perfusion Flows</th>
<th>Critical tissue levels of Doxorubicin found</th>
<th>Critical perfusate levels of Doxorubicin found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnston / 1983</td>
<td>45 min</td>
<td>100 ml/min</td>
<td>levels &gt; 10-20 ug/g demonstrated irreversible lung damage</td>
<td>Not available</td>
</tr>
<tr>
<td>Minchin / 1985</td>
<td>50 min</td>
<td>300 ml/min</td>
<td>Levels at 28.8 ug/g demonstrated severe lung damage</td>
<td>Initial levels of 11 ug/ml demonstrated severe lung damage.</td>
</tr>
<tr>
<td>Baciewicz / 1991</td>
<td>45 min</td>
<td>210 ml/min</td>
<td>Levels &gt; 20 ug/g were associated with lung injury</td>
<td>Levels &gt; 7 ug/ml were associated with lung injury</td>
</tr>
<tr>
<td>Furrer / 1997</td>
<td>15 min</td>
<td>70-120 ml/min</td>
<td>Tissue levels of 21.9 ug/g were associated with hemorrhagic lung edema.</td>
<td>Not available</td>
</tr>
<tr>
<td>Dos Santos / 2013</td>
<td>4 hours</td>
<td>450-550 ml/min</td>
<td>Levels of 61.82 ug/g were considered safe</td>
<td>Levels of 23 ug/ml were considered safe</td>
</tr>
</tbody>
</table>

Table 6 – Comparison of the previous large animal IVLP studies with Doxorubicin.

We recognize that the extent of the IVLP studied here (4 hours) may seem long for clinical application. However, we found differences in the final levels of Doxorubicin when we compared 2 and 4 hours of IVLP followed by 4 hours of reperfusion. The duration of IVLP is a concept that needs to be addressed, because it may be important for the studies with chemotherapy, where an increment in the perfusion time was associated
with higher final tissue levels, as previously reported in the case of Doxorubicin [163] and Cisplatin [164].

The long-term IVLP (4 hours) actually demonstrated for how long we can perfuse a lung within a closed circuit in a safe way. This may also open opportunity for other localized therapies that require longer exposure window such as gene therapy[165]. However, we do recognize that the time of IVLP may be variable for different drugs, like Melphalan[166]. Thus, pharmacokinetic studies with different drugs will need to be performed and the time of perfusion may be modified according to these data.

One aspect that demonstrates the importance of a safe strategy to perform IVLP is related to the perfusion flows during IVLP. An interesting quote by Johnston et al. states that “physiologic flow rates (and possibly ideal drug distribution) could not be obtained in single lung perfusions without exceeding normal PA pressures”[123]. In fact, the majority of IVLP studies done so far established the perfusion flow rate based on “physiologic” PA pressures, which were pre determined before the start of IVLP. We think that this strategy may have hidden the injuries induced by the perfusion circuit, as smaller perfusion flows could be used in order to minimize PA pressure levels, reducing the chance of developing lung edema.

In contrast to this, in our study the perfusion flow was never adjusted to pre determined PA pressure levels. It was calculated according to the CO of the animal and the resultant PA pressure levels were recorded. Using these calculations, usual flows to the left lung were 450 to 550 ml/min depending on animal weight and estimated CO. These flows were 2-4 times higher than previous flows described in large animal studies where IVLP
was performed with Doxorubicin [95,97,98,109]. Surprisingly, normal levels of PA pressure were found (10-15 mmHg), even after the administration of standard doses of chemotherapy. This can be explained by the protective IVLP strategy, which allowed higher perfusion flows under physiologic PA pressure and PVR, protecting the perfused lung. Of note, the calculation of the flows was performed according to the low-flow strategy used for the EVLP protocol, but we can state that, in an IVLP setting, these flows can actually be considered high flows, when compared to previous studies (Table 6).

In regards to pharmacokinetics, we found levels of chemotherapy in lung tissue that were also higher than previous reports. The novel IVLP strategy may contribute to this as it avoids lung injury during IVLP, suggesting that injury could limit the absorption of chemotherapy by healthy lung tissue.

Optimal distribution of chemotherapy in lung tissue could also be attributed to the protective IVLP strategy as it avoids hypoxia of the perfused lung during IVLP. The high oxygenation levels found in the outflow of the perfusion circuit, which reflects the alveolar PO2, supported this. Thus, we do not induce hypoxic pulmonary vasoconstriction and this phenomenon may lead to disparities in the perfusion of localized areas of the lung [5] and could contribute to a suboptimal distribution of chemotherapy in lung tissue.

We demonstrated that IVLP can be performed with high-dose chemotherapy for an extended perfusion time with minimal lung injury, as shown by physiologic parameters,
Importantly, our study showed that we were able to perfuse a lung for an extended perfusion time, administering higher levels of Doxorubicin under higher perfusion flows (and normal PA pressure levels) and achieve higher final tissue levels of chemotherapy evenly distributed in different areas of the lung tissue.

While most studies done in the past associated the use of IVLP for patients with unresectable lung metastases, we believe that the efficacy of this procedure can be increased if the micrometastastic disease is considered as the target. In studies done with patients with unresectable lung metastases, the levels of chemotherapy in normal lung tissue were higher than the intra-tumoral levels obtained and attempts to increase the dose of chemotherapy were invariably associated with lung injury. In these studies, the equivalence between tumor levels and normal lung levels usually demonstrated discrepancies, whereas an inverse correlation between tumor size and intra-tumoral levels of chemotherapy after IVLP was found both experimentally and clinically when smaller tumors were analyzed. This issue was confirmed in a clinical study where IVLP aimed to target micrometastastic disease, and for this purpose, only lung metastases smaller than one centimeter were used for pharmacokinetics purposes. In this setting, equivalent levels of chemotherapy were found in normal lung tissue and tumor [124]. This was confirmed experimentally, where higher concentrations of chemotherapy were found in smaller tumors in comparison to tumors that were larger [131]. With our IVLP strategy, we showed that it is possible to safely administer higher levels of chemotherapy than previously reported. Thus, the association of our strategy with the intention to target
micrometastatic disease after the resection of lung metastases may be the ideal clinical scenario for IVLP.

To our best knowledge, this is the first study that describes a technique that allows a perfusion of a lung in situ, for an extended perfusion time and without acute lung injury, even after the administration of standard doses of chemotherapy used in clinical practice. Nevertheless, we recognize that the absence of long-term follow up is a limitation of this study, but we also emphasize that the acute lung injury following IVLP needs to be tackled, considering that this injury that is found immediately after IVLP is usually maintained after 24-72 hours [98]. Thus, acute lung injury is probably the critical step to be overcome in this setting.

Our model focused on many physiologic measures that weren’t explored previously. In general, the large animal studies performed to date have focused on long-term effects of IVLP. Signs of acute lung injury were correlated with the outcomes of the animals in the long-term. However, even an injured lung is able to overcome the most critical acute phase injury and gradually improve the function over time[104]. The gap lies in the fact that probably a less effective IVLP was being performed, usually with smaller doses of chemotherapy, smaller perfusion time and single agents.

We also found that bronchial circulation needs to be properly addressed during long-term IVLP, as this blood flow may dilute the chemotherapy in the perfusion circuit, leading to lower perfusate and tissue levels of chemotherapy.

Taking advantage of the expertise with EVLP, we performed three perfusions with human lungs rejected for clinical transplantation due to infection. The overarching goal
of these human lung perfusion studies was to analyze the pharmacokinetics of Doxorubicin in a unique preclinical model, using solid phase micro extraction (SPME)[166]. This technique may allow for an optimized and faster measurement of chemotherapy in perfusate and lung tissue, and will be of great importance in future studies. These three EVLP cases allowed us to identify that, once the donor lung has preserved function, EVLP with chemotherapy was stable with 75 mg/m$^2$ and 225 mg/m$^2$ of Doxorubicin, suggesting that the human lung can be more resistant to the injury than the porcine lung. In the case where the donor had poor function, the addition of Doxorubicin 150 mg/m$^2$ demonstrated severe lung injury, according to the pattern aforementioned.

Currently, many studies are being performed in order to better select candidates for lung metastasectomy. However, many patients with lung metastases remain within an area of uncertainty about the best management, especially since local recurrence occurs at a very high rate. We believe that this work is an initial effort that can open new possibilities to tackle exactly this problem, demonstrating that a modified strategy to perfuse a lung in situ for an extended perfusion time allowing the administration of conventional doses of chemotherapy exclusively to an organ and achieving drug levels with minimal injury will contribute to a better management of this population of patients. IVLP will potentially have an impact not only in the prognosis of the current candidates for lung metastasectomy, but also will open new possibilities for “borderline” candidates for surgical treatment or those with high risk of recurrence, extending the indication of a “curative” resection of lung metastases.
6 Conclusion
The objectives of this research project were to (1) develop a large animal model of IVLP with a modified technique that allowed an extended perfusion time and minimized perfusion-related injury, attenuating lung injury and (2) to analyze the safety of the administration of high-dose chemotherapy to the lungs using the IVLP strategy developed.

First, we translated the concepts of the EVLP model, using a protective perfusion/ventilatory strategy and were able to demonstrate that IVLP can be performed for an extended perfusion time and without perfusion-associated injury.

We then demonstrated that this IVLP strategy allowed a safe administration of higher doses of chemotherapy and achieved higher levels of chemotherapy in perfusate and lung tissue in comparison to previous studies. We also demonstrated that an extended perfusion time was possible, leading to higher final levels of Doxorubicin in lung tissue. The administration of a mixed scheme of drugs was also demonstrated to be safe using our IVLP platform. Furthermore, the limits of the dosage of Doxorubicin administered in this IVLP model were described.

Finally, recognizing that animal studies will never reproduce the human setting, we take advantage of our experience with EVLP and perfused human lungs with high-dose chemotherapy in a tentative to analyze the effects of high dose chemotherapy in human tissue.
In summary, this work provides evidence of the safety administration of high-dose chemotherapy regarding acute lung injury. This is an important step towards to make the IVLP technique a safer and more effective method to deliver chemotherapy to the lungs for patients with pulmonary metastases, minimizing the effects of the chemotherapy in lung tissue. Our plan is to then design a Phase I human clinical trial based on the data obtained from this study.
7 Future directions
An animal model that allows perfusion of a lung within a closed circuit without additional injury is an important advance as it attenuates acute lung injury, which was the main problem associated with previous IVLP studies.

Even considering that our IVLP results were excellent with respect to acute lung injury, we believe that the long-term effects of this platform need to be evaluated. The injury that happens after IVLP is critical during the first hours after perfusion and even considering that this pattern of injury is usually maintained for the next 24-72 hours, survival studies using the same model will give relevant answers to our project. For example, the analysis of long-term toxicity to the lungs and also potential late systemic toxicities.

A reliable platform to study IVLP is also an opportunity to reassess concepts that were studied in the past, like hyperthermic perfusions and different regimens of chemotherapy that may target other types of cancer like colorectal carcinomas.

The principles established in this project will be also adapted to a small animal model of IVLP. This is particularly important because models of lung metastases can be induced in rats and efficacy studies will be possible.

Targeted therapies can be potentially studied using our approach. In fact, one of the weaknesses of these therapies is the proper delivery to the specific site of disease. Hence, our platform tackles exactly this problem, enabling the isolation of the target-organ, and the delivery of the therapy exclusively to the desired site. Gene therapy was already studied in the past and we believe concept must be reassessed, benefiting not
only from the approach developed, but also from the extended perfusion time, allowing 
more exposure of the organ to the targeted therapy.

We are also working on a SPME for future pharmacokinetic studies. Briefly, this 
technique allows measurement of the doses of chemotherapy in lung tissue and perfusate 
using small needles. The potential benefit of this technique is that a quick assessment of 
the levels of drugs in lung tissue and perfusate and also the characteristic to separate the 
amount of drug which is circulating from the drug that actually is inside the tissue will be 
possible, translating in the actual levels of tissue drugs, as opposed to HPLC, which 
doesn’t discriminate between both.

Hence, considering our promising results, a Phase I study to analyze the safety of this 
approach in patients with metastatic sarcomas to the lungs will be developed in order to 
prevent recurrence after lung metastasectomy, targeting residual micrometastatic disease. 
We do hope that this project will be an initial effort in order to open new curative 
possibilities for patients with pulmonary metastatatic disease.
8 Dissemination of Work
8.1.1 Presentation in Meetings

Santos PR, Iskender I, Machuca T, Hwang DM, Keshavjee S, Waddell T, Cypel M.


8.1.2 Publications

Santos PR, Iskender I, Machuca T, Hwang DM, Keshavjee S, Waddell T, Cypel M.

*Modified IVLP Allows for Prolonged Perfusion without Acute Lung Injury.* Accepted for publication in the Journal of Thoracic and Cardiovascular Surgery.
9 Appendices
9.1 Doxorubicin (Adriamycin)

Doxorubicin is an anthracycline agent which binds to DNA-associated enzymes, intercalating with DNA pairs, stopping the process of replication.

It is extensively metabolized in the liver and up to 50% of drug is eliminated in the feces via biliary excretion. Urinary excretion accounts for 4-5% of the administered dose.

The main side effects are:

Hematologic: leukopenia, anemia, thrombocytopenia, neutropenia.

Cardiologic: side effects can present acutely (tachycardia or arrhythmias) or chronically (asymptomatic reduction in the left ventricular ejection fraction, arrhythmias, congestive heart failure). Heart failure is probably the most important complication of this drug, and the probability to develop this complication is estimated around 1-2% at a cumulative dose of 300 mg/m². The risk increases proportionally to increments in the doses (3-5% at 400 mg/m²; 5-8% at 450 mg/m² and 6-20% at 500 mg/m²). Thus, it is not recommended to exceed a maximum cumulative dose of 550 mg/m² of Doxorubicin.

In relation to the lungs, no toxicity is reported.
9.2 Ifosfamide

Ifosfamide is a nitrogen mustard alkylating agent, and these drugs exert their cytotoxic effects via transfer of their alkyl groups to various cellular constituents. Alkylating of DNA within nucleus creates intra and interstrand cross links, and probably represents the major interactions that lead to cell death. The major site of alkylation within DNA is the N7 position of guanine. Although alkylating agents are not cell cycle specific, cells are most susceptible to alkylation in late G1 and S phases of the cell cycle.

It is metabolized primarily in the liver and eliminated by the kidneys.

The main side effects are:

Neurotoxic: somnolence, confusion, hallucinations, depressive psychosis, seizures and, in some instances, coma.

Nephrotoxic: hemorrhagic cystitis is frequently encountered, and Ifosfamide should always be accompanied by the use of Mesna (uroprotective treatment). Renal parenchyma and tubular necrosis also have been reported.

Hematologic: leucopenia, thrombocytopenia and anemia

In relation to the lungs, interstitial pulmonary fibrosis has been reported in fewer than 1% of patients treated with larger doses of alkylating agents for prolonged period, although not reported in patients treated with Ifosfamide.
10 References


Pastorino U, Buyse M, Friedel G, Ginsberg RJ, Girard P, Goldstraw P. Long-
term results of lung metastasectomy: prognostic analyses based on 5206 cases.

[29] Cerfolio RJ, Bryant AS, McCarty TP, Minnich DJ. A prospective study to
determine the incidence of non-imaged malignant pulmonary nodules in
patients who undergo metastasectomy by thoracotomy with lung palpation.

al. A 10-year single-center experience on 708 lung metastasectomies: the
evidence of the "international registry of lung metastases". J Thorac Oncol

Pulmonary metastases from soft tissue sarcoma: analysis of patterns of diseases

resection of metastatic sarcoma: prognostic factors associated with improved

[33] Stanelle EJ, Christison-Lagay ER, Wolden SL, Meyers PA, La Quaglia MP.
Pulmonary metastasectomy in pediatric/adolescent patients with synovial

[34] D'Adamo DR. Appraising the current role of chemotherapy for the treatment of

[35] McCormack PM, Burt ME, Bains MS, Martini N, Rusch VW, Ginsberg RJ.
Lung resection for colorectal metastases: 10-year results. Arch Surg


[66] Weksler B, Bruce N, Lenert JT, Burt ME. Isolated single-lung perfusion with


[103] Van Der Elst A, Oosterling SJ, Paul MA, Vonk AMA, Sparidans RW, van der


159


[130] Hengst Den WA, Van Putte BP, Hendriks JMH, Stockman B, Van Boven W-


1987;93:11–8.


