Numerical and experimental models of pulmonary interstitial edema development

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April, 2016
“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie
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Abstract

Lung edema is a life-threatening condition occurring when fluid extravasates from pulmonary vessels and capillaries into interstitium and into alveoli. The cause of pulmonary edema can be ascribed either to an increase in pulmonary capillary pressure or an increase in micro-vascular permeability due to fragmentation of the extracellular matrix and of endothelial glycocalix. Several degrees of pulmonary edema have been identified; an early phase, named interstitial edema, involves fluid accumulation in the peri-alveolar septal interstitium, while the late phase causes alveolar flooding thus compromising lung diffusing capacity. In normal conditions, lung is well protected against the development of pulmonary edema, due to interstitial tissue rigidity and vaso-active responses aiming to divert blood flow to non-edematous and well perfused regions. Mechanisms triggering fluid accumulation in edemagenic conditions involves modification of both vascular compartment and interstitial fluid balance.

The aim of this PhD Thesis is to develop an integrated approach, combining innovative modelling frameworks and new experimental data, in order to exploit the adaptive response of lung to edemagenic conditions and evaluate the interaction among the different physiological variables.

A model based on several well-known phenomena of capillary network perfusion was included in a set of equations describing the interstitial fluid balance to provide an integrate view of the main mechanisms involved in pulmonary edema development. Alveolar mechanics was studied by in-vivo microscopy in normal and edemagenic conditions in a rabbit lung model; arteriolar and capillary adaptive response to hypoxia administration was evaluated and vascular mechanics was estimated by applying the model to experimental images. Finally, MRI sequences were applied to investigate the regional distribution of lung edema and evaluate both the sensitivity of the imaging technique to moderate lung density change and the different response to hydraulic and permeability-induced edema.

An integrate approach bridging the gap between modelling and experimental data allows to fully and more deeply characterize the mechanisms involved in edema formation.
Summary

Lung primary function is to allow gas exchange between the alveolar air space and the capillary blood coming from pulmonary artery. To accomplish this task, lung presents a wide exchange surface provided by the complex airway tree structure and a thin exchange barrier, made by extracellular matrix, epithelial and endothelial cytoplasm. Fluid accumulation in the air-blood barrier reduces its diffusing capacity and affects the whole organ functionality. The interstitial compartment is extremely dry in normal conditions, due to combined effect of a high tissue rigidity provided by a well-organized structural components and an effective lymphatic drainage action. Lung edema can be caused by an increased capillary pressure, favouring filtration from the vascular towards the tissue compartment, for example during heavy exercise or in condition of diastolic dysfunction of the left ventricle, retrogradely increasing pulmonary pressure and generating the so-called hydraulic edema; permeability-induced edema, on the other hand, is characterized by an increase of capillary permeability due to disruption of the physical structure of the pores in the microvascular membrane.

In many disease conditions, edema oscillates between the hydraulic and permeability-induced type. In fact, an increased pressure and thus blood flow may produce an increase of endothelial gaps due to shear stress applied on the capillary glyocalix; on the other side, permeability-induced edema may produce a redistribution of blood flow to non-edematous regions causing an increase of vascular pressure in different lung areas. Furthermore, increase of extra-vascular lung water and thus of interstitial pressure may compress lung capillaries, providing a passive mechanism of protection against further development. A well-known non-cardiogenic edemagenic condition is hypoxia, which provokes an inflammatory state enhancing fluid permeability and a vaso-active response characterized by two contrasting phenomena, namely arteriolar vaso-constriction and capillary recruitment. Understanding the complex mechanisms underlying the physiological response to hypoxia may help to identify the mechanical variables responsible for fluid accumulation and provide a deeper insight into the pathophysiology of lung edema.

A complete evaluation of the functional adaptive response of capillary compartment to edemagenic condition involves the use of different imaging technique to image the in-vivo adaptive response to perturbative stimuli, such as hypoxia administration or saline injection. The aim of this Thesis is to provide a theoretical framework to analyze the interaction between lung capillaries and interstitium during the onset of edema, in order to systematically review and integrate all the mechanisms involved in fluid balance control; the model should be calibrated and compared with morphometric and
hemodynamic data. For this purpose, a network capillary model was built, based on previous formulations and adapted to rabbit lung morphometric data; capillary recruitment and flow patterns were interpolated and included in a model of extra-vascular lung water. The modeled alveolar-capillary unit (ACU) can be used to describe several edemagenic condition and compute the dynamic changes of the variables involved in the control. Furthermore, it can be applied to experimental images to derive indications about microvascular variables from morphometric measurements.

The specific objectives of this project are:

1) To develop a computational model for describing the main factors acting in the development of lung edema.

2) To adapt and/or develop algorithms for imaging analysis for automatic and semi-automatic processing of in-vivo microscopy images.

3) To apply in-vivo microscopy and imaging analytical programs to analyze lung subpleural alveolar mechanics in rabbit normal lungs.

4) To apply in-vivo microscopy and imaging analytical programs to analyze vascular adaptation to hypoxia in rabbit models of edematous lungs.

5) To study global and regional adaptation of lung in two mouse models of edema (hypoxia and saline injection) by $^1$H MRI.

6) To integrate experimental data with model for a deeper understanding of physiologic mechanisms involved in edema formation.

Chapter 1 - Computational micro-scale model of control of extra-vascular water and capillary perfusion in the air blood barrier.

This Chapter presents the physiological basis to understand the principal factors acting at the air-blood barrier in the control of extra-vascular lung water. Air-blood barrier is made by a thin region mainly constituted by interstitial cell cytoplasm, and a thick region made mainly by extracellular matrix, bearing the structural components providing the mechanical properties of lung parenchyma. Alveolar septal surface is covered by a dense capillary network (having 5-6 µm as average diameter), fed by terminal arterioles and collecting into pulmonary venules; pulmonary circulation is characterized by a reduced pressure range compared to the systemic one and capillary compartment has been found to represent the main resistance site for blood flow but also of recruitment. Capillary recruitment reduces the network resistance by enrolling new parallel pathways and increases the surface area for both gas exchange and for micro-vascular filtration. Alveolar septa are subject to structural variations during breathing, depending on the
force balance between tissue elastic properties and surface tension; this dynamic equilibrium may be altered by excess fluid in edemagenic conditions. Fluid extravasation towards interstitium and alveolar space may increase due to an increase in capillary pressure or in permeability and may be triggered by different events. Lung is well protected against the development of edema thanks to an effective lymphatic action and a rigid tissue structure buffering small increases in extra-vascular lung water.

**Chapter 2 - Computational micro-scale model of control of extra-vascular water and capillary perfusion in the air blood barrier.**

In this Chapter, the principal modelling approaches used to characterize the perfusion at the capillary level are reviewed and discussed. Fung's model of pulmonary capillary bed described the interstitial-capillary compartment as an elastic sheet made by pillars, representing interstitial posts and separating free spaces representing capillary lumen. This approach has the advantage of offering a lumped-parameter characterization of the whole capillary network, but was questioned by electron image microscopy, showing a tubular structure for the micro-vascular pulmonary meshwork. The network model requires a higher computational cost since it has to determine blood flow and pressure for each capillary segment, but offers the advantage of being applicable to real in-vivo microscopic images and thus to provide information about capillary recruitment and blood flow for each vascular segment. In the second part of the Chapter, previous modelling studies of the pulmonary interstitial lung regions are discussed; Fung's sheet proposal was integrated into an interstitial fluid model involving the study of fluid movement within interstitium, described by the Darcy law. The most recent formulation for fluid control is based on a 1-st order algebraic system of 4 equations: 1) revised Starling equation accounts for micro-vascular filtration, 2) Kedem-Katchalsky equation describes the solute exchange which modulates oncotic gradient, 3) interstitium is described as a capacitance able to accumulate fluid, 4) lymphatic drainage depends on interstitial liquid pressure, on lymphatic resistance and on lymphatic pumping activity. The presented perfusion and the fluid control models represent the basis for the successive chapter.

**Chapter 3 - Computational micro-scale model of control of extra-vascular water and capillary perfusion in the air blood barrier.** A computational model of a morphologically-based alveolar capillary unit (ACU) in the rabbit was developed to relate lung fluid balance to mechanical forces between capillary surface and interstitium during development of interstitial edema. We hypothesized that positive values of interstitial liquid pressure $P_{liq}$ impact on capillary transmural pressure and on blood flow. ACU blood flow, capillary recruitment and filtration are computed by modulating vascular and interstitial pressures. The model was validated against experimental data of $P_{liq}$ increasing from ~ -10 (control) up to ~ 4 cmH$_2$O in two conditions, hypoxia and
collagenase injection. For hypoxia exposure, fitting data required a linear increase in hydraulic conductivity $L_p$ and capillary pressure $P_C$, that fulfilled the need of increase in oxygen delivery. For severe fragmentation of capillary endothelial barrier (collagenase injection), fitting required a rapid increase in both hydraulic and protein permeability, causing ACU de-recruitment, followed by an increase in $P_C$ as a late response to restore blood flow. In conclusion, the model allowed to describe the lung adaptive response to edemagenic perturbations; the increase in $P_{\text{aq}}$, related to the low interstitial compliance, provides an efficient control of extra-vascular water, by limiting micro-vascular filtration.

Chapter 4 - From morphological heterogeneity at alveolar level to the overall mechanical lung behavior: an in vivo microscopic imaging study. In 6 male anesthetized, tracheotomized and mechanically ventilated rabbits, we imaged subpleural alveoli under microscopic view (60x) through a “pleural window” obtained by stripping the endothoracic fascia and leaving the parietal pleura intact. Three different imaging scale levels were identified for the analysis on increasing stepwise local distending pressure ($P_{ld}$) up to 16.5 cmH2O: alveoli, alveolar cluster and whole image field. A semi-automatic algorithm to segment alveolar profile is presented, based on the integration of a method allowing to find alveolar centers from a raw image and a shortest-path-based algorithm to estimate the optimum alveolar border. Alveolar profiles were traced, clusters of alveoli of similar size were identified through a contiguity-constrained hierarchical agglomerative clustering analysis and alveolar surface density (ASD) was estimated as the percentage of air on the whole image field. Alveolar area distributions were remarkably right-skewed and showed an increase in median value with a large topology-independent heterogeneity on increasing $P_{ld}$. Modelling of alveolar area distributions on increasing $P_{ld}$ led to hypothesize that absolute alveolar compliance (change in surface area over change in $P_{ld}$) increases fairly linearly with increasing initial alveolar size, the corollary of this assumption being a constant specific compliance. Clusters were reciprocally interweaved due to their highly variable complex shapes. ASD was found to increase with a small coefficient of variation (CV< 25%) with increasing $P_{ld}$. The CV of lung volume at each transpulmonary pressure was further decreased (about 6%). The results of the study suggest that the considerable heterogeneity of alveolar size and of the corresponding alveolar mechanical behavior are homogenously distributed, resulting in a substantially homogenous mechanical behavior of lung units and whole organ. Experiments for this and the following chapter were conducted in collaboration with a research group of the Dipartimento di Medicina Sperimentale, Università di Milano Bicocca, led by Prof. Giuseppe Misericocchi.

Chapter 5 - Regulation of alveolar micro-vascular perfusion and interstitial fluid balance. In-vivo microscopy of closed-chest subpleural regions was performed on 4 male anesthetized, tracheotomized and mechanically ventilated rabbits, by using the
same approach described in Chapter 4. After 1-hour of control, hypoxia (12% O₂) was administered via mechanical ventilation for two hours. Distribution arterioles were imaged and their caliber was automatically measured by adapting a segmentation algorithm. Corner vessels and alveolar profile were also segmented. Hemodynamic analysis revealed, as expected, a clear vasoconstriction in the first 40 minutes of hypoxia administration, identified as PHASE I, but followed by an increase of capillary perfusion in the following hour, named PHASE II; corner vessels followed the same mechanical behaviour. Alveolar mechanics was affected by hypoxia administration, first at end-expiration and then also at end-inspiration. Finally, a 3D version of the model proposed in Chapter 3 was applied to experimental images in order to estimate the hemodynamic variations occurring during the hypoxia treatment. The optimal combination of arteriolar inlet pressure and venular outlet pressure was found by matching the variations between capillary perfusion pressures and corner calibers measured by imaging. Results from this integration between experimental data and modelling confirm, for PHASE I, the expected result of arteriolar vasoconstriction but suggest also an important role of venular constriction, which contribution is still under debate. In PHASE II, a more heterogeneous behaviour was found, reflecting biological variability among networks. Furthermore, PHASE I was characterized by a strong septal derecruitment, at odds with previous data. The results of the study suggest a two-phase behaviour of the adaptive response to hypoxia and confirm the heterogeneity of this behaviour for the late phase of acute edema development.

Chapter 6 - Regulation of alveolar micro-vascular perfusion and interstitial fluid balance. MRI is an useful imaging approach to map lung edema regional development and identify edema sub-clinical interstitial phase. The aim of this study was to study the sensitiveness of two image sequence, namely gradient echo (GRE) and a custom ultrashort time echo (UTE) to measure small fluid accumulation in lung parenchyma and possibly to derive indications about the regional distribution of mild interstitial edema. 36 mice were divided in four groups. 8 were immediately sacrificed to obtain a control value for the wet-to-dry ratio; 8 mice were imaged under isofluorane anesthesia, 8 underwent hypoxia administration (10% O₂) and 12 saline injection. Imaging was performed at baseline, immediately after treatment and half an hour after the start treatment; thus, two time points after baseline were obtained. Fluid accumulation was obtained by comparing slice by slice lung parenchymal proton density normalized to proton density of a reference rod, for both GRE and UTE sequences. Wet-to-dry ratio increased only for saline injection group; no statistically significant variation was found for or the hypoxic group, while a slight trend towards higher values, even if not significant, was found for the isofluorane group. GRE confirmed the increase of lung density, but only for the first time point. Furthermore, by evaluating the regional distribution of lung proton density in the caudo-cranial direction, fluid accumulation showed a greater increase in the upper region of the lung. The custom UTE sequence
found similar, but no significant, results for saline group; however, the greater resolution of the UTE images allowed to obtain information about the gravity gradient: fluid accumulation was higher in the gravity dependent region of the lung. Results of this study confirms a good sensitivity of MRI sequence for monitoring lung density; gradient evaluation revealed a regional variation of extra-vascular lung water density due to difference in hydraulic pressure and/or in capillary density. In order to investigate this aspect, two ACUs, differing by capillary density, were compared to explain regional distribution of lung edema. Experiments were performed at Cincinnati Children's hospital in collaboration with the CPIR group of Prof Jason Woods.

**Chapter 7 - Conclusions**

In this chapter, the focus and the main results of this Thesis are presented, discussing the specific aims of each study. Future perspectives of this PhD project are also introduced.
# Glossary

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$A_{\text{liq}}/A$</td>
<td>Ratio between the area of capillary surface over which liquid interstitial pressure acts and total capillary surface</td>
</tr>
<tr>
<td>$A_s/A$</td>
<td>Ratio between the area of capillary surface over which solid interstitial pressure acts and total capillary surface</td>
</tr>
<tr>
<td>ACU</td>
<td>Alveolar capillary unit</td>
</tr>
<tr>
<td>ASD</td>
<td>Alveolar surface density</td>
</tr>
<tr>
<td>$C$</td>
<td>Interstitial compliance ($ml \cdot ml^{-1} \cdot cmH_2O^{-1}$)</td>
</tr>
<tr>
<td>$C_c$</td>
<td>Capillary compliance ($m \cdot cmH_2O^{-1}$)</td>
</tr>
<tr>
<td>CC</td>
<td>Corner capillaries</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>Conc$_c$, Conc$_i$</td>
<td>Plasma and interstitial protein content ($kg/m^3$)</td>
</tr>
<tr>
<td>CV</td>
<td>Collecting venules</td>
</tr>
<tr>
<td>$D_i$</td>
<td>Diameter of the $i$-th capillary segment ($m$)</td>
</tr>
<tr>
<td>$D_m$</td>
<td>Diameter of the smallest vessel that a RBC can pass through ($m$)</td>
</tr>
<tr>
<td>GRE</td>
<td>Gradient echo sequence</td>
</tr>
<tr>
<td>$H_i$</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>$J_l$</td>
<td>Lymphatic drainage flow ($m^3/s$)</td>
</tr>
<tr>
<td>$J_{sl}$</td>
<td>Rate of lymphatic drainage of interstitial proteins ($kg/s$)</td>
</tr>
<tr>
<td>$J_{sv}$</td>
<td>Micro-vascularprotein extravasation rate ($kg/s$)</td>
</tr>
<tr>
<td>$J_v$</td>
<td>Filtration flow ($m^3/s$)</td>
</tr>
<tr>
<td>$L_i$</td>
<td>Length of the $i$-th capillary segment ($m$)</td>
</tr>
<tr>
<td>$L_p$</td>
<td>Hydraulic conductivity ($m \cdot s^{-1} \cdot cmH_2O^{-1}$)</td>
</tr>
<tr>
<td>$m$</td>
<td>Number of capillary segments in the ACU</td>
</tr>
<tr>
<td>$n$</td>
<td>Number of junctions in the ACU</td>
</tr>
<tr>
<td>$N_{\text{BC}}$</td>
<td>Number of boundary conditions</td>
</tr>
<tr>
<td>$N_{\text{Voronoi}}$</td>
<td>Number of Voronoi points of capillary network</td>
</tr>
<tr>
<td>$P_c$</td>
<td>Average ACU capillary luminal pressure ($cmH_2O$)</td>
</tr>
<tr>
<td>$P_{Ci}$</td>
<td>Capillary luminal pressure of the $i$-th junction ($cmH_2O$)</td>
</tr>
<tr>
<td>$P_{el}$</td>
<td>Elastic pressure of lung parenchyma ($cmH_2O$)</td>
</tr>
<tr>
<td>$P_{ld}$</td>
<td>Alveolar local distending pressure ($cmH_2O$)</td>
</tr>
<tr>
<td>$P_{liq}$</td>
<td>Interstitial liquid pressure ($cmH_2O$)</td>
</tr>
<tr>
<td>$P_{lps}$</td>
<td>Local pleural pressure ($cmH_2O$)</td>
</tr>
<tr>
<td>$P_{out}$</td>
<td>Lymphatic outlet pressure ($cmH_2O$)</td>
</tr>
<tr>
<td>$P_p$</td>
<td>Lymphatic pumping pressure ($cmH_2O$)</td>
</tr>
<tr>
<td>$P_y$</td>
<td>Pressure of surface tension ($cmH_2O$)</td>
</tr>
<tr>
<td>PS</td>
<td>Surface area-protein permeability product ($m^3/s$)</td>
</tr>
<tr>
<td>$P_{tm}$</td>
<td>Capillary transmural pressure ($cmH_2O$)</td>
</tr>
</tbody>
</table>
\( Q \) ACU input blood flow \((m^3/s)\)
\( Q_i \) Blood flow of the \( i \)-th capillary segment \((m^3/s)\)
\( Q_p \) Interstitial protein content \((kg)\)
\( Q_{RBC} \) Red blood cells \((RBCs/s)\)
\( r_{recr} \) Percentage of capillary length contributing to gas/fluid exchange
\( r_i \) Radius of the \( i \)-th capillary segment \((m)\)
\( R_L \) Lymphatic resistance \((cmH_2O \cdot s \cdot m^{-3})\)
\( R_{seg_i} \) Resistance of the \( i \)-th capillary segment \((cmH_2O \cdot s \cdot m^{-3})\)
\( S_{alv} \) Alveolar surface area \((m^2)\)
\( SC \) Septal capillaries
\( S_{cap} \) Capillary exchange surface area \((m^2)\)
\( TA \) Terminal arterioles
\( UTE \) Gradient echo sequence
\( V \) Interstitial fluid volume \((m^3)\)
\( V_{cap} \) Global capillary volume \((m^3)\)
\( V_{interst} \) Interstitial volume \((m^3)\)
\( V_0 \) Initial interstitial fluid volume \((m^3)\)
\( \overline{\Delta \rho} \% \) Average increase of normalized lung density
\( \mu_{app} \) Apparent blood viscosity \((Pa \cdot s)\)
\( \mu_c \) Cytoplasmic viscosity \((Pa \cdot s)\)
\( \mu_p \) Plasma viscosity \((Pa \cdot s)\)
\( \pi_L \) Plasma protein oncotic pressure \((cmH_2O)\)
\( \pi_T \) Interstitial protein oncotic pressure \((cmH_2O)\)
\( \sigma_f \) Protein reflection coefficient
\( \tau \) Interstitial thickness \((m)\)
1. Physiology of the air-blood barrier in the lung
1.1 Overview of lung anatomy at the level of air-blood barrier

The lung primary function is gas diffusion from air to venous blood. To ensure an efficient gas exchange, a large air-blood interface surface extends in lung parenchyma by a complex alveolar-capillary interacting structure. Alveoli are the most peripheral unit of the pulmonary airway tree, which is composed by a conducting and a respiratory region. The conducting zone consists of trachea, bifurcating into primary bronchi which in turn give rise to secondary and then tertiary bronchi. This subdivision continues in an asymmetrical way till the terminal bronchioles, while structural changes occur, such as a reduction in cartilage support structures and increase in smooth musculature. In fact, bronchioles lack cartilage support and have a complete layer of circular smooth muscle. The respiratory zone is identified by the presence of alveoli; respiratory bronchioles are the first structure to be alveolated, leading to alveolar ducts and then to terminal clusters of alveolar acini, composed of alveoli.

At the alveolar level, the difference in partial pressure oxygen $P_{O_2}$ between oxygenated air and venous blood drives oxygen absorption by pulmonary capillaries. In human lung, $O_2$ supply by air is guaranteed by a branched system of airways connecting almost 1000 million alveoli to the trachea [1][2]. Pulmonary gas diffusion occurs at the level of the alveolo-capillary membrane, which represents an efficient gas exchanger due to its reduced thickness and high surface area (130 m$^2$ of alveolar surface in humans [2]).

Alveolar septal interstitium

The air blood barrier is made of "thick" and "thin" portions (Figure 1.1). The latter is composed by alveolar epithelial cells, capillary endothelial cells and an intervening fused basement membrane [3], while the former is mainly composed by cytoplasm of interstitial cells. The harmonic mean, given by the weighted average of thickness between the thin and thick portions, reflects the resistance to gas diffusion and is about 0.6 µm in human lung [4]. Type-I epithelial cell occupy most of alveolar surface, being thin and oblong, while round and smaller type-II alveolar cell are stem cells able to differentiate into type-I and responsible of synthesis and secretion of surfactant.

An important molecular component of the basement membrane and of cell surface is heparan sulfate, a sub-family of proteoglycans which contribute to the sieving property of the endothelial wall, modulating the permeability of the microvasculature endothelium to plasma components [5].

In the "thick" portion, the alveolar and capillary cells delimit the pulmonary interstitium, a thin compartment made of a fiber system serving as a scaffold; the extracellular matrix (ECM) of interstitium consists mostly of collagen I and III, elastin fibers, providing rigidity to lung tissue [6] and hyaluronan and chondroitin sulphate, another important proteoglycan sub-family. These molecular components favour lateral
binding of collagen and elastin assembly [7]; furthermore, hyaluronan and proteoglycans in the ECM regulate extra-vascular water by binding excess fluid to form gel-like structures. An efficient control of the fluid content within the extracellular space is required to guarantee an optimal gas exchange; interstitial water is regulated by the balance between the filtration from the capillaries and the removal by the lymphatic system. Collagen and elastin bundles concentrate in the entrance ring of the alveolus to provide structural support. Other important cell types present in alveolar septal wall are fibroblasts, which are involved in extracellular matrix protein synthesis but also, being contractile, in interstitial rigidity and regulation of capillary blood flow, macrophages and rare nerve fibers. The arithmetic interstitial thickness amounts to 1.63 µm in human lung [8], where about 60% is cellular contribution and the rest is structural (collagen and elastin).

Figure 1.1 Electron microscopy appearance of the air-blood barrier in rabbit lung. Capillary lumen containing erythrocytes are separated from the air compartment by a thin region, responsible for diffusion, which thickness is represented by the harmonic thickness. The thick region is populated by interstitial cells (both type I and II) and extracellular matrix. Reprinted from [9].

1.2 Pulmonary vascular system

Beside ventilation, the second important factor guaranteeing an optimal oxygen diffusion is blood perfusion, which is accomplished by the pulmonary vascular system. This is characterized by lower pressures and lower resistance with respect to the systemic circulation; moreover, direct measurements of micro-vascular pressure in rabbit lungs
highlighted that the most resistive component is represented by capillaries [10], whereas systemic arteries provide the greatest pressure drop along systemic blood pathway.

**Pulmonary arterial system**

Human pulmonary arteries carrying venous blood from right ventricle are organized into 17 orders from the main pulmonary artery (order 17) to the smallest pre-capillary arteriole (10-15 µm), with an average increase in diameter ratio of about 65% [11]. The arterial caliber varies during the respiratory cycle; vessels in the alveolar compartment (mainly capillaries and smaller arterioles and venules) are compressed by positive alveolar pressure during lung inflation, while smaller extra-alveolar vessels, mechanically coupled to lung parenchyma, expand and/or lengthen during lung distension. The arterial wall is composed by an adventitial layer and a thin intima, composed by non-fenestrated endothelial cells. The adventitia is composed by extracellular matrix, fibroblasts and an innervating network, which extends to small intra-acinar vessels. Adventitial composition varies throughout arterial orders; larger arteries are mainly elastic and muscular while smaller arteries tend to have fragmented or absent elastic lamina but preserve smooth muscle cells even within the pulmonary acinus. High heterogeneity is present in the degree of muscularity of smaller arterioles, but only smaller arterioles (less than 120 µm) may be totally non-muscular; the ability of contraction is an important regulatory aspect of blood flow in the capillary compartment.

**Capillaries**

At odds with the systemic circulation, where the most resistive compartment is the arterial branching system, capillaries and small arteries were found to mostly contribute to pulmonary vasculature resistance, as confirmed by both micropuncture measurements [10] and by a recent approach based on resistance decomposition through a windkessel estimation of diastole decay in pulmonary arterial and venous pressure [12].

The origin of capillary networks may be highly heterogeneous; e.g., they can either arise from smaller arterioles, or emerge laterally from a large artery. Capillary networks are topologically organized as a dense interconnection of short segments occupying mainly alveolar septal wall but also some regions adjacent to bronchovascular bundles, an anatomical structure composed by an airway, an artery and often a vein and a lymphatic vessel (Figure 1.2). Diameter of capillary segments ranges from 5 to 8 µm, with subpleural capillaries being 30% larger and 10% less dense than intra-acinar segments. The pathway of blood from arterioles to venules ranges from 250 to 850 µm. Squamous endothelial cells delimit capillary lumen, the total surface of which amounts to 130 m² in human lung. Due to the reduced presence of gap junctions, pulmonary endothelium is characterized by very low hydraulic permeability, representing the extravasation flow per unit of pressure gradient and per unit of exchange surface.

Besides septal capillaries, at the intersection of three adjacent alveolar walls, blood is conveyed by corner capillaries, which are defined as those extraseptal vessels occuring
at the intersection of at least three alveolar septa. Corner capillaries represent a parallel system to the septal network preserving blood flow in condition of elevated alveolar pressure; in fact, while lung inflation tends to compress septal capillaries, it stretches alveolar walls and thus corner vessels. The importance of corner vessels has been highlighted in an in-vivo microscopic study of subpleural vasculature, which found that in condition of high alveolar pressure (above mean pulmonary arterial pressure) corner vessels [13] guarantee blood flow in alveolar compartment.

![Figure 1.2](image)

**Figure 1.2** Casts of pulmonary artery and capillaries imaged by electron microscopy. Note the complex capillary meshwork, more packed in the alveolar septa (right bottom panel, scale bar indicates 50 µm) and less dense in the subpleural region (right top panel, scale 50 µm) and in the perivascular region (left panel, black bar at lower left indicates 100 µm). Reproduced from [14].

The surface of the gas exchanger may vary in conditions requiring an increase in oxygen delivery, such as exercise. In fact, in-vivo microscopy of subpleural region showed that pulmonary capillaries are the main site of recruitment during conditions of increased blood flow and/or pressure [15], even if recruitment can occur also in the arteriolar compartment [16]. Capillary bed recruits by both distension and opening of previously unperfused capillaries, providing a reduction of total pulmonary vascular resistance (PVR), due to the presence of multiple parallel pathways for blood. Changes in surface area available for fluid exchange, corresponding to the capillary recruited surface area, were found to correlate with changes of pulmonary vascular resistance [16]. The meaning of capillary recruitment has been questioned by [17] who injected albumin-coated nano-particles in rabbit lung and found presence of the tracer in the whole capillary network; this result allows to hypothesize that it should be distinguished between plasma recruitment and red blood recruitment, since these two blood components may take different paths.
Capillary volume is a function not only of vascular fluid dynamics conditions but also of lung mechanics. In particular, an increase in surface tension alters alveolar mechanics and produces a reduction in capillary volume and compliance [18], at a greater extent for higher lung volumes, as a consequence of both a direct effect of surface tension on capillaries bulging in the alveolar lumen and of septal tissue retraction.

**Pulmonary veins**

In human, 15 orders of pulmonary veins branch similarly to pulmonary arteries. With respect to the latter, pulmonary veins are less muscularized and contain more collagen; furthermore, large extra-alveolar walls present cardiac myocytes which extend from the left atrium and which activity may modulate venous tone. Regulation of pulmonary pressure in capillary compartment may be actively accomplished also by venous sphincters, muscular structures found in lung of smaller rodents [19].

### 1.3 Alveolar mechanics and imaging techniques

The lung functional unit is represented by the alveolar duct, a corridor opening on several chambers named alveoli. The understanding of the mechanisms of lung expansion at the micro level is required to optimize ventilatory strategy and therapeutic approaches in mechanical ventilation, which should avoid both alveolar collapse and overdistension. However, alveolar mechanics has not been completely elucidated yet. The main forces acting at the alveolar level are tissue elastic force $P_{el}$ and surface tension $P_\gamma$ at the interface between air and the thin liquid layer which lines alveolar epithelium. The sum of these two collapsing forces equilibrates alveolar pressure which at functional residual capacity (FRC) is atmospheric in static conditions. An important stabilizing factor which impedes alveolar collapse is alveolar surfactant which is synthesized and secreted by alveolar epithelial type-II cells and tends to reduce surface tension; this effect is maximal when alveolar surface is minimal during the respiratory cycle, since surfactant is more densely packed along the air-liquid interface, while it is at minimum during the peak of inspiration (2).

Several non-exclusive mechanisms of alveolar expansion have been proposed, including isotropic alveolar expansion, recruitment of previously collapsed alveoli, alveolar shape change and alveolar "uncrumpling", akin to a paper bag being crumpled and uncumpled [20].

Pioneer histological studies strengthened the hypothesis of isotropic expansion, but their results were consistently biased by the fixation technique used [21]. Some in-vivo microscopy studies based on open-chest animal surgery suggested that subpleural alveoli tend to change size minimally during ventilation ([22], [23]), thus suggesting that
alveolar duct recruitment may contribute to lung micromechanics. Main limitations of this approach are the open-chest model, which alters physiological mechanical conditions and the fact that only subpleural alveoli are analyzed, thus impeding an extrapolation of indications to the remaining lung.

A recent work [24] based on in-vivo microscopy of subpleural alveoli in mechanically ventilated rats, analyzed alveolar mechanics in control and surfactant-deprived lung; this perturbation is required to estimate the parameters of a lung alveolar spatial model and establish by Bayesian inference the most likely micromechanical model. Simulation experiments suggested two different possible behaviours: isotropic balloon-like alveolar expansion or largest increase in sac mouths size with stiffer alveolar walls. Both these two solutions suggest two different ventilatory strategies: while a more distensible alveolus would allow to use greater mechanical pressure without damaging lung tissue, a more rigid alveolar wall will require to carefully calibrate ventilation pressure to avoid lung injury. Differentiating between these two hypotheses would require to investigate the dynamics of alveolar sac mouths for which in-vivo microscopy of lung subpleural region is not a proper technique.

The challenge of real time imaging of internal alveolar dynamics has recently been afforded by using a technique named tracking X-ray microscopy [25] [26], based on the use of phase contrast and strongly collimated synchrotron X-rays, which are highly penetrating. By investigating both the apices and the largely moving regions at the base of lung in live rats, tracking of individual alveoli was possible; alveolar inflation was found to be quite heterogeneous even among adjacent alveoli, revealing some degree of asynchrony even within the same alveolar sac. A slightly greater expansion was found for the alveoli at the base but the average increase in alveolar area resulted to be 6.7%; this fact made the authors suggest that the central channel of the alveolar duct may be greatly involved, thus confirming a non-isotropic expansion at the micro level.

The contribution of alveolar ducts has recently been put forward by using an ingenious technique based on the analysis of the diffusion of hyperpolarized $^3$He within alveolar ducts [27]; this indirect estimate allowed to derive in-vivo indications for human lung and suggested that lung inflation consists of a large recruitment and an "accordion-like" expansion" of alveolar ducts rather than by isotropic expansion of alveoli.

**Mechanical interdependence between capillaries and alveoli**

Alveolar mechanics is strictly correlated to capillary perfusion, since it can be altered in edemagenic conditions and can alter pulmonary vascular response to edemagenic stimuli. An interesting microscopic study on isolated and perfused lungs [28] [29] used the micropuncture technique to instill liquid into individual alveoli and found that alveolar edema reduces the size of liquid-filled alveoli, but do not alter their compliance. More interestingly, air-filled alveoli adjacent to liquid-filled ones presented an increase in size, due to the difference in liquid pressure between the edematous alveolus and the air-filled one, but a reduction in compliance, probably related to overexpansion injury.
Chapter 1

and to the stiffening of tissue at higher volume; this mechanical interaction between adjacent alveoli has been often invoked as a key factor providing high heterogeneity in individual alveolar behaviours after perturbative stimuli.

On the other side, [30] demonstrated that setting high tidal volume and pressures in mechanical ventilation may produce pulmonary edema, and may cause microcapillary rupture with increased capillary permeability and gas exchange deterioration. Another important in-vivo microscopic study [31] investigated whether altered alveolar mechanics due to surfactant deactivation may influence the primary physiologic response of lung vasculature to alveolar hypoxia, namely pulmonary vasoconstriction. As detailed in paragraph 1.6, pulmonary vasoconstriction is an adaptive mechanism through which the lungs divert blood flow from poorly aerated regions or regions receiving less oxygen to normally ventilated regions, in order to obtain an adequate perfusion-ventilation match. Surfactant-deprived alveoli were more unstable and tended to collapse during the respiratory cycle. Even if with both normal and altered alveolar mechanics hypoxia caused vasoconstriction of larger vessel (diameter > 30 µm) and vasodilation of part of the smaller ones, unstable alveoli increased micro-vascular diameter despite an increase of the vasoconstrictor response to hypoxia. The effect of corner vessel expansion due to increased surface tension in surfactant-deprived alveoli is similar to the effect of lung inflation in normal conditions: during lung expansion, septal capillaries tend to collapse and corner capillaries tend to expand due to the mechanical interdependence.

1.4 Micro-vascular fluid exchange

Micro-vascular fluid exchange is governed by Starling's equation, which states that fluid flow from capillary lumen to interstitial space is proportional through the fluid filtration coefficient $K_f$ to the driving pressure gradient, composed by an hydrostatic gradient ($P_c - P_{liq}$) and a colloid osmotic pressure gradient ($\pi_c - \pi_i$) acting across the micro-vascular membrane.

$$ Jv = K_f \left( P_c - P_{liq} - \sigma f \left( \pi_c - \pi_i \right) \right) $$

Eq. 1.1

$K_f$ depends both upon the hydraulic conductivity of all the pores in the membrane and on the membrane surface area, which could change together with capillary perfusion. $P_c$ represents capillary pressure, while interstitial fluid pressure $P_{liq}$ is sub-atmospheric in normal conditions. Interstitial oncotic pressure $\pi$ is proportional to the concentration of interstitial proteins, whose presence is due to the fact that semi-permeability of micro-vascular membranes is imperfect: in fact, the endothelial barrier allows some plasma proteins to reach the interstitial space. The degree of leakiness to proteins is quantified by the osmotic reflection coefficient $\sigma$ and in the lung lies in the range 0.4-0.7 [32].
Actually, all solutes contribute to oncotic pressure difference, proportionally to the permeability of vessel membrane to them; smaller solutes have smaller osmotic reflection coefficient and therefore contribute less to the total oncotic pressure difference. Experiments evaluating the role of protein concentration in micro-vascular fluid equilibrium mainly related to albumin, the most abundant protein found in blood plasma [33].

Starling model elicits to assume that arterial end of single capillaries tends to filter out fluid due to a highest capillary pressure, while the venous end tend to reabsorb fluid. However, experiments on a single frog mesenteric capillary [33] showed that by quickly reducing capillary pressure from a high value to low values (~10 cmH$_2$O) absorption from tissue space to capillary, but at steady-state, by maintaining capillary pressure low for some minutes, there is only a small net filtration. Therefore, for steady-state behaviour, no re-absorption was found for low values of capillary pressure while filtration occurred only for capillary pressure overcoming ~20 cmH$_2$O.

Figure 1.3 Deviation from classic Starling equation (short dashed line) of experimental data (dotted points, taken from [34]). 3D and 1D modelling of the endothelial glycocalix accounting for local oncotic pressure gradients fits more closely experimental data. Adapted from [35].

This deviation from the linear relationship expressed by Eq. 1.1 and shown in Figure 1.3 has been explained by considering that linear relationship does not consider the complex pathway that fluid must travel from plasma to interstitium: the current three-dimensional model proposed by Michel and Weinbaum ([36], [37]), reduced to one dimension in [35], is composed by a surface glycocalyx matrix layer and an intercellular cleft region, with the former being the principal barrier for plasma proteins. In fact, the "small pores" of the plasma membrane of non-fenestrated capillaries, as those found in lung and/or mesentery, accounting for 90-95% of the hydraulic permeability, are too large to be the molecular filter of albumin [36]. Therefore, glycocalyx determines the effective oncotic force for water flow across capillary endothelium; as a consequence, Starling equation
applies locally across the surface matrix layer rather than globally among plasma and tissue. Referring to the experiments of Michel and Philips [33], this model allows to explain the different behaviours in transient and steady-state conditions: in transient conditions, interstitial oncotic pressure \( \pi_i \) remains low since proteins are not able to diffuse from capillary lumen to tissue and absorption is favoured; in steady-state conditions, the entire protein concentration gradient is experienced across the surface glycocalyx but the oncotic force in the cleft is sufficient to oppose the filtration pressure. The result is that at steady state no net filtration flow is observed across the surface glycocalyx, thus correlating with the threshold-like behavior found by [33].

**Lymphatics**

Pulmonary lymphatic system represents a unidirectional circulatory system which main function is to drive excess lung fluid and proteins from the interstitial/alveolar space back to circulation. Beside the control of interstitial fluid, pulmonary lymphatic vasculature contributes to lung fluid clearance at birth [38] and to inflammatory and immune response [39]. The most peripheral region of lymphatic system is represented by capillaries made by fenestrated and one-way passively valved endothelial cells, anchored with filaments to adjacent tissue; at this level, lymph formation depends mainly on external tissue deformations, such as due to breathing, rather than to active lymphatic contractions. Once formed, lymphatic fluid is driven back to circulation by means of contractive units named "lymphangions", separated by unidirectional valves impeding back flow; smooth muscle cells in the outer walls of lymphatics provide the pumping action of lymphangions which contract sequentially driven by an electrical pacemaker activity. Two different lymphatic systems contribute to lung fluid balance: interstitial space beneath visceral pleura is drained by a complex system of prelymphatics, made by sheets of connective tissue, and reservoir-like structures, converging into conduit lymphatics; the second lymphatic system guaranteeing inner lung dryness drives fluid through saccular and tubulo-saccular lymphatics mainly surrounding small blood vessels and airways and converge into tubular conducting lymphatics running along bronchovascular bundles and/or interlobular septa. Lymph flow from the inner lung flows towards the hylum, while lymph from interlobular septa is collected by pleura. Given the mechanical interaction between lung and lymphatics, lymph flow is correlated to ventilation and in particular to tidal flow [40].

A recent work [41] analyzed lymphatic morphology in different regions of normal human lung and found no significant difference in lymphatic density between subpleural, interlobular and intralobular vessels. As to intralobular regions, lymphatic vessels are mainly peri-vascular rather than peri-bronchiolar while very few vessels were found within alveolar walls. Relative to size, vessel area decreases from central lung to periphery. Peripheral lymphatics mainly consist of small vessels surrounding arterioles; it has been found a ratio of one lymphatic every two arterioles having a diameter less than 50 µm [42]. Therefore, apparently, fluid equilibrium at the acinar interstitial level
may not rely on an efficient drainage action by lymphatics due to their low density; however, fluid flow from interalveolar septa to lymphatic system is guaranteed by the pressure difference between the pressure in the septal interstitial space and that in the perivascular interstitial space, which is more negative and more populated by terminal lymphatics [43]. Furthermore, mechanisms guaranteeing tissue dryness include also the already described low vessel permeability and very low tissue compliance, as detailed in next paragraph.

**Tissue compliance and interstitial fluid pressure**

The complex structure of extracellular matrix (ECM) of lung interstitium described in paragraph 1.1 provides a high rigidity of lung interstitium to fluid accumulation; low compliance means a great increase of interstitial fluid pressure for a small increase in fluid volume. This "tissue safety factor" allows to reduce Starling gradient and thus net filtration during the first phase of interstitial edema. Furthermore, as described above, proteoglycans may bind excess fluid to form gel-like structures. However, with edema progression, tissue rigidity decreases due to partial fragmentation and degradation of structural elements, mainly proteoglycans. During hydraulic edema, due to increased micro-vascular pressure and developed by intravenous injection of saline solution, extra-vascular water produces tissue stress and weakening of proteoglycan non-covalent bonds, with a consequent increase in tissue compliance. In lesional edema, developed by elastase injection and due to enzymatic activity, there is a degradation of heparan sulfate leading to an increase in micro-vascular permeability [6]. In both cases, ECM metalloprotease are activated and contribute to tissue degradation.

1.5 Interstitial lung edema

Interstitial lung edema is an acute condition characterized by a fluid accumulation within the interstitial lung compartment, due to increased filtration from the vascular compartment. It may develop mainly from an increase in the pressure gradient sustaining filtration from capillaries to interstitium or in the weakening of the endothelium hampering filtration of fluid and proteins. In the first case, Starling pressure gradient can be altered by both an increase in hydraulic capillary pressure, due to left-sided heart failures, or a decrease in osmotic gradient, with the former being more clinically significant; for this reason, this condition is known as cardiogenic pulmonary edema. In the case of increased permeability pulmonary edema, increased fluid filtration is due to the fragmentation of the sieving alveolar-capillary barrier.
Cardiogenic pulmonary edema

Increased pulmonary capillary pressure may be due to increased venous resistance such as for mitral stenosis or left atrial myxoma [44], or left ventricular failure due to systolic or diastolic dysfunction of the left ventricle. This condition can occur also after vigorous exercise, such as for athletes, since increased metabolic demand rises pulmonary arterial pressure; exercise-induced pulmonary edema has been questioned, since those athletes developing symptoms of lung edema may be produced by underlying cardiac pathology and because an increase in pulmonary arterial pressure does not necessarily increase capillary pressure, due to capillary recruitment [45]. Anyway, bronchoalveolar lavage fluid (BALF) analysis highlighted the presence of red cells for athletes subject to strenuous activities, even if at a subclinical level [46]. Lung is well protected by initial interstitial hydrostatic edema, since, being usually permeability of capillary endothelium unaffected, interstitial concentration of large proteins such as albumin decreases, as the interstitial tissue matrix becomes more porous, thus increasing the osmotic gradient which reduces filtration. A chronic high capillary pressure, however, may deteriorate endothelium due to mechanical stress and thus increase capillary permeability and foster edema development.

Two main phases of lung edema can be identified: first, excess fluid extravasated in the low compliant interstitial space surrounding capillary compartment but is removed by lymphatic drainage; further increase in fluid accumulation is accommodated by the more compliant tissue compartment surrounding arterioles, bronchioles and venules (bronchovascular bundles). In the second phase, excess fluid can no more accumulate in the interstitial compartment and provokes a fragmentation of alveolar-capillary barrier with consequent alveolar flooding, resulting in a decrease of blood oxygenation and worsening of left ventricular functioning.

Non-cardiogenic pulmonary edema

Non-cardiogenic pulmonary edema (NCPE) is characterized by an increase in endothelial permeability and filtration of fluid in the interstitial and alveolar compartment and may be caused by a direct (inhalation of toxic gases, injection of vasoactive substances, pancreatitis) or indirect insult (infectious septicemia, thoracic trauma) [47]. Unlike cardiogenic pulmonary edema, the interstitial phase of fluid accumulation is often by-passed due to a rapid fragmentation of alveolar epithelium, with subsequent alveolar flooding. The loss of integrity of the sieving barrier causes also a decrease in the reflection coefficient, which thus impairs the protective effect of the oncotic gradient across the membrane. The mechanical compression of the capillary and arteriolar vasculature may also produce an increase in hydraulic pressure and thus a worsening of fluid-dynamic stress of epithelium.
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Other causes of pulmonary edema

Pulmonary edema may be generated by a concomitant increase of capillary pressure and permeability. Neurogenic pulmonary edema is caused by sympathetic overstimulation causing pulmonary vasoactive constriction, with an increased blood flow into the lungs and a stiffer left ventricular myocardium, producing a transient rise of hydraulic pressure disrupting endothelium and epithelium. High altitude pulmonary edema (HAPE) is a life-threatening disease occurring at altitudes higher than 2500 m [48]; even if this condition is characterized by symptoms of non-cardiogenic edema, such as BALF rich in large proteins and markers of inflammation, due to the effect of hypoxia on capillary permeability, it is associated with pulmonary hypertension and increased capillary pressure [48], maybe due to hypoxic venous vasoconstriction or to heterogeneity of hypoxic arteriolar vasoconstrictor response which causes regional overperfusion of some areas with subsequent increase of blood flow and $P_c$. Pulmonary capillary permeability was found to be in normal range. Thus, HAPE results to be a multifactorial acute condition involving both an increase of capillary permeability and of vascular pressures.

Many other conditions may generate pulmonary edema [47]. Antidepressants administration provokes increased pulmonary capillary permeability; the same effect may be caused by calcium channel blockers which impair prostaglandin protective action on vascular cellular integrity and cause an alteration of the perfusion-ventilation ratio or by the allergic reaction to radio-contrast media. Anaesthesia treatments may produce upper airway obstruction due to laryngospasm which decreases pleural pressure and thus increases the hydrostatic Starling gradient. Lung overperfusion, produced e.g. after a pneumonectomy which increases blood flow in the remaining lung, may lead to fluid overloads with subsequent increased filtration flow; a similar behaviour is observed in ischemia-reperfusion induced injuries. Hypoalbuminemia and generally hypoproteinemia are correlated and may be a cause of lung edema and Acute Respiratory Distress Syndrome (ARDS), due to effect of a decreased oncotic gradient on Starling filtration.

Hypoxia mediated pulmonary edema

Hypoxia affects pulmonary fluid equilibrium by means of two main pathways: the first one is related to the cerebral neurologic insult [49] and the other to the vascular adaptive response [50]. In particular, cerebral-mediated pulmonary edema may be the result of two concurrent phenomena: central hypoxia increases intracranial pressure and cerebral flow and this leads to an increase of sympathetic nervous system activation through catecholamine secretion; on the other side, increased production of reactive oxygen species (ROS) may enhance both pulmonary capillary permeability and sympathetic activation [49].

Relative to hemodynamics, hypoxia causes recruitment of pulmonary capillaries, thus increasing surface area for gas exchange. This mechanism preserving lung diffusion capacity and blood oxygenation requires some hemodynamic modifications which have
not been fully elucidated yet. The first possible reason of an increased pulmonary capillary blood volume, experimentally found in different species, from dog [51] to humans [52], is the short-term increase in cardiac output and in pulmonary arterial pressure, which elicits a passive recruitment of the capillary network due to its distensibility. Another possible mechanism relies on an increased left atrial pressure due to a compromised left ventricular diastolic function, causing an increase of capillary pressure and thus of capillary bed recruitment. Finally, hypoxia triggers vascular vasoconstriction of both pre-capillary and post-capillary vessels by the modulation of the inhibition of postassium channels in pulmonary vascular smooth cells. This adaptive response provides a defensive mechanism to hypoxic insult in the fetus, who thus diverts blood to the systemic circulation, preserving his vital functions; in the adult, vasoconstriction is thought to be a functional adaptation aiming at diverting blood in well perfused areas, thus preserving the ventilation/perfusion match. In particular, arterial vasoconstriction reduces capillary pressure, perfusion and recruitment, while venous vasoconstriction increases capillary pressure downstream. A recent work [50] performed a retrospective statistic analysis of hemodynamic parameters in healthy humans before and after hypoxic mixture (12.5%) administration and found, by a multiple regression analysis, that the hypoxia-induced increase in capillary volume may be only in part explained by the increase in cardiac output, in pulmonary artery pressure or in left ventricular diastolic function; these results strengthened the role of hypoxic post-capillary vasoconstriction for capillary recruitment.

Measuring lung edema
The most used clinical diagnostic and prognostic index related to pulmonary edema is the extra-vascular lung water (EVLW), for which the gold standard is post-mortem gravitational measurement of lung water content (wet to dry ratio). Several techniques have been applied to obtain an indirect estimate of EVLW in vivo in ill patients. Chest radiography allows to estimate the distribution of fluid accumulation but is based on semi-quantitative scores assigned by radiologists and requires high expertise to distinguish pulmonary edema indications from other lung microstructural features (e.g. ground glass opacification, atelectasis) [53]. MRI and CT scanning could be the best option but are expensive and impractical for bed-rest patients. The most widespread solution in clinical practice is the indicator solution technique, which can use a single or a double indicator [54]. The latter case, representing the gold standard for intrathoracic water volume measurement, involves the injection into a venous access point of two saline solutions of a indocyanine dye and of a cooled glucose bolus and their concentrations are measured in aorta, obtaining cardiac output and mean transit time of each bolus. EVLW is obtained as the difference between the intrathoracic blood volume (ITBV) measured as the product of the transit time and of cardiac output for the thermal plus dye bolus minus the ITBV measured only by indocyanine green, which does not interfere with EVLW. Single indicator is instead based only on thermal dilution.
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technique; by computing the slope of the exponential decay time of the thermal concentration, it is possible to obtain the EVLW by introducing two coefficients estimated by linear correlation with the gold standard [55]. Many studies found a good agreement between the single and the double indicator technique [55] [54], so that the first one has been applied in clinical practice through a commercial system (PiCCO, Pulsion Medical Systems, Munich, FRG).

Invasiveness of the injection technique has led to explore other approaches to estimate lung water volume. Thoracic ultrasonography has shown the potentiality to be a non-invasive tool to diagnose early pulmonary edema even before the appearance of gas diffusion impairment and consequent blood deoxygenation. Normal lung is unechoic due to the air content and ultrasound images show horizontal parallel "A-lines" due to reverberation artefacts within the pleural space, but in condition of increased water lung content, vertical "B-lines" appear due to the change in acoustic impedance, increasing ultrasound penetration. B-lines have been found to correlate with radiological lung scores in dyspnoecic and cardiological patients; in hemodialytic patients, B-lines reduced post-treatment and they are sensitive also to athletes' small changes in extra-vascular lung water and oxygen saturation during intense activities and after recovery. However, ultrasounds offer still a semi-quantitative estimate of EVLW and suffers from user-dependency; B-line artefact can be revealed also in other diseases such as pulmonary fibrosis, and may depend on expiratory pressure and respiratory cycle. A good review of ultrasound estimation of EVLW can be found in [56].

Other promising techniques are microwave reflectometry imaging and electrical impedance monitoring. Reflectometry imaging system is based on an array of antennas transmitting highly unidirectional microwave (750-1000 MHz) pulses; the intensity of the backscattered microwave beams increases in case of increased fluid density thus allowing, after a preprocessing aimed to reduce background noise, a sensitive quantitative imaging of fluid density inside the lung [57]. Electrical impedance tomography (EIT) is an imaging technique reconstructing the map of the conductivity within three-dimensional structures, based on the injection of electrical currents and detection of voltages by means of an electrode array. EIT is an established tool for thoracic imaging but, at present, provides relative and not absolute imaging due to the ill-posed inverse problem solving. Anyway, EIT imaging at different position of the subject allows to highlight possible gravitational ventilatory inhomogeneities related to the development of pulmonary edema. A good correlation was found between the EIT-derived index of pulmonary edema and the gravimetric measurement in [58].

No-reflow phenomenon

Few studies has analyzed the effect of the mechanical interaction occurring between lung interstitium and pulmonary capillary compartment. A passive compressive effect of tissue edema is micro-vascular occlusion and has been observed and validated by experimental observations of the no-reflow phenomenon occurring in post-ischemic
skeletal muscles [59]. Ischemia-reperfusion produces enzymatic and inflammatory response, with increased neutrophil production and subsequent neutrophil adherence mainly to post-capillary venules; furthermore, leukocyte adhesion provokes an increase in micro-vascular permeability with subsequent increase in interstitial fluid volume (edema formation) and, for low compliant tissues such as muscle fascial sheath or lung interstitium, increase in interstitial pressure. This mechanical condition may be a secondary factor producing micro-vascular no-reflow since it tends to compress capillaries. The alteration of vascular pressure and flow distribution due to the compressive effect of interstitial liquid is also evident in tumours, where the high central interstitial fluid pressure diverts blood from the internal region of tumour from more external paths; this is relevant for radiotherapy, since, despite the highly permeable vessels, the core of tumour is not sufficiently perfused to be reached by drugs [60].

1.6 Aims and outline of the Thesis

The presented review of the patho-phsyiological mechanisms underlying pulmonary edema development leaves some open questions: in particular, the relevance of the role of the mechanical interaction between the pulmonary capillary and the interstitial compartment has not been determined and no available model, based on morphometric data, applicable to experimental in-vivo images and able to describe that interaction, has been found in literature. The adaptive response of the pulmonary vascular compartment to hypoxia, a well-known edemagenic condition, is still under debate, since it involves several constrasting phenomena (e.g. vasoconstriction and capillary recruitment). Moreover, interstitial edema is still considered a subclinical condition, preceding the onset of the life-threatening alveolar edema; therefore, it will be useful to verify imaging sensitivity to this mild pathological condition. Finally, relative to pulmonary parenchymal mechanics, there is still no consensus on the main mechanism of alveolar expansion, possibly affecting capillary perfusion recruitment.

Based on these open problems, the objective of the present PhD project is to implement modelling approaches and integrating imaging experimental data to quantify and elucidate the main mechanisms providing protection against fluid accumulation in edemagenic conditions. The proposed model, validated and integrated with experimental data, should become a useful basis to describe the pathogenesis of severe lung edema in a variety of conditions ranging from exhaustive exercise to pathological conditions, including adult and neonatal respiratory distress syndromes.

The specific aims of this project are:

1) To develop a computational model for describing the main factors acting in the development of lung edema.
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2) To adapt and/or develop algorithms for imaging analysis for automatic and semi-automatic processing of in-vivo microscopy images.
3) To apply in-vivo microscopy and imaging analytical programs to analyze lung subpleural alveolar mechanics in rabbit normal lungs.
4) To apply in-vivo microscopy and imaging analytical programs to analyze lung interstitial and vascular adaptation to hypoxia in rabbit models of edematous lungs.
5) To study global and regional adaptation of lung in two mouse models of edema (hypoxia and saline injection) by 1H MRI.
6) To integrate experimental data with model for a deeper understanding of physiologic mechanisms involved in edema formation.

The Thesis is structured as follows.

Chapter 1 introduces the physiological variables involved in the fluid balance of pulmonary interstitial compartment and the main pathways for pulmonary edema development. A brief review of theoretical modelling of both pulmonary capillary perfusion and interstitial fluid balance is discussed in Chapter 2. In Chapter 3, the mechanical interaction between capillary and interstitial mechanics is analyzed by a comprehensive model, integrating morphological data and physiological phenomena involved, for both animal and human model. In Chapter 4, alveolar mechanics is studied by in-vivo microscopy in a closed-chest animal model, in order to assess heterogeneity in mechanical behaviour at different scale levels. Chapter 5 shows the effect of an edemagenic stimulus, i.e. hypoxia administration, on arteriolar, capillary and alveolar mechanics and bridges the gap between experimental data and modelling. Finally, chapter 6 describes the effect of hypoxia administration and of saline injection on mouse lung based on MRI analysis; modelling is applied to interpret experimental results.
2. Theoretical background of modelling lung perfusion and lung fluid balance
2.1 Fung sheet model

The two main model-oriented morphological descriptions of pulmonary capillary network are based on different geometrical assumptions: the first one describes the capillary mesh as a network of interconnecting tubes and has been proposed by Weibel [61], while the second one simplifies capillary topology as a sheet structure and is due to Fung ([62], [63], [64], [65]). First histological results based on gelatin or formalin injection lead Fung to hypothesize that capillary mesh can be correctly described by a sheet model, composed by two parallel surfaces of capillary endothelia connected by cells and interstitial tissue; this model is often indicated as a "parking garage", where the capillary space represent the garage free space and the intervening cellular and interstitial posts are the "garage pillars". The most important parameter of the model is the sheet thickness, describing the blood pressure relationship and the mechanical interaction between blood and capillary endothelia.

Fung geometrical model is made of hexagons having a "pillar" in the center of each polygon; with respect to the tube model, this simplified description has the advantage to better describe the curvature of the junctions between capillary segments but introduce a sharp connection between pillars and sheets. In [65], the value of the ratio between capillary luminal volume and interstitial volume found in an experimental preparation of silicon-filled cat lung vasculature, ranging around 90%, supports the sheet-flow model, which presents an higher capillary density than the tubule model. Furthermore, capillary sheet thickness was found to increase not more than 0.4 µm from 7.4 to 7.8 µm when intravascular pressure increases from 15 to 25 cmH_2O.

Pressure-flow relationships in sheet-and-post model are based on Navier-Stokes equations [62]; pressure gradient is proportional to fluid velocity by means of two multiplicative coefficients, respectively depending on geometrical variables (length and sheet thickness) and on topology of posts (interpostal distance and post diameter). The first coefficient was derived by geometric deductions, while the second one was experimentally determined through an artificial test bench. Sheet elasticity was described by a linear relationship between sheet thickness and transmural pressure, but sheet compliance can be derived to depend on forces acting on the sheet itself; in particular, an increased surface tension, e.g. occurring during lung inflation, reduces sheet compliance and thus blood flow [63]. The solution of Navier-Stokes equation can be obtained by solving the harmonic equation \( \nabla^2 \varphi = 0 \), where the stream function \( \varphi \) is proportional to the sheet thickness elevated to the fourth power. By hypothesising a simplified alveolar structure made by a square sheet with an arteriolar input and a venular output, the average blood flow \( Q \) per unit width can be estimated to be directly proportional to the fourth power of sheet tickness and inversely proportionally to the sheet length \( L \) [63]:
Theoretical background of modelling lung perfusion and lung fluid balance

\[ Q = \frac{1}{4\mu f L \alpha} [(h_0 + \alpha \Delta p_{art})^4 - (h_0 + \alpha \Delta p_{ven})^4] \quad (Eq. 2.1) \]

where \( \mu \) is blood viscosity, \( f \) is the coefficient related to post topology, \( \alpha \) is sheet compliance, \( h_0 \) is the sheet thickness at null transmural pressure and \( \Delta p_{art} = p_{art} - p_{atv} \) and \( \Delta p_{ven} = p_{ven} - p_{atv} \) are the transmural pressures at the arteriolar and venular access points, respectively. Eq. 2.1 can be developed to highlight the proportionality between flow and the arteriolar-venular pressure gradient:

\[ Q = \frac{1}{4\mu f L} (p_{art} - p_{ven})[(h_0 + \alpha \Delta p_{art})^3 + (h_0 + \alpha \Delta p_{art})^2 (h_0 + \alpha \Delta p_{ven}) + (h_0 + \alpha \Delta p_{art})(h_0 + \alpha \Delta p_{ven})^2 + (h_0 + \alpha \Delta p_{ven})^3] \quad (Eq. 2.2) \]

The proportionality coefficient in brackets, named conductance, is also function of arteriolar and venular pressure, since these pressure influence sheet thickness. The total flow can be obtained by integrating all the streamlines between the arteriole and the venule, having a mean length \( \bar{L} \), in the vascular space \( V \):

\[ Q = \frac{V}{4\mu f L^2} (h_{art}^3 + h_{art}^2 h_{ven} + h_{ven}^2 h_{art} + h_{ven}^3) (p_{art} - p_{ven}) \quad (Eq. 2.3) \]

The standard Poiseuille equation for cylindric tube with diameter \( d \) is:

\[ Q = \frac{\pi d^4}{128\mu L}(p_{art} - p_{ven}) \quad (Eq. 2.4) \]

Therefore, the difference between Eq. 2.2 and Eq. 2.3 stands only on the different geometrical proportionality factor and on the dependence of the conductance on the pressure variables.

Fung and Sobin’s sheet model provided an useful tool to model and investigate pulmonary microcirculation with a simplified approach but was based on images of optical microscopy lacking adequate resolution and focus depth for applying modelling to them. First scanning electron images of rat lung capillary networks [14] showed that pulmonary microcirculation presents two different types of organizations: subpleural and perivascular microcirculation tend to develop into long tubules within a low-density meshwork, while internal alveolar networks present a double-layered structure with frequent interconnections within the alveolar septum. The main finding was that capillary length is often greater than capillary diameter, thus supporting the model of interconnecting tubes originally proposed by Weibel.

These results opened a debate in early 1990's between the authors (Guntheroth et al. [66]) and the sheet model's authors (Fung and Sobin [67]). In particular, Guntheroth criticized the sheet-and-post paradigm because of several aspects, both morphologic and functional: the assumption of a single layer of capillaries, even if supported by some evidence from imaging, does not allow to explain how interstitial fluid can migrate to lymphatics if the accumulation occurs within the posts; septal elastic elements form a reticulum rather than focal points; posts would prevent capillary collapse and de-recruitment, which can occur in conditions of elevated alveolar pressure; a double layer
of capillaries would be optimal for gas diffusion allowing endothelia to be directly exposed to air.
Fung and Sobin defended their approach questioning the double layer morphology and observing that their model could anyway be easily extended to describe different capillary topologies [67]. Anyway, network model has been put forward as a valid alternative and has been proposed to describe the pulmonary capillary system.

2.2 Network model

The network model was applied to pulmonary microcirculation by [68] and [69], but it had already been used to describe the less dense systemic microcirculation [70]. As described, the model is made by tubes representing capillaries within an alveolar septum, interconnecting at junctions; in [68], each capillary segment is characterized by a height $h$, depending on transmural pressure:

$$h = h_0 + (P_{C_i} - P_A) \quad (Eq. 2.5)$$

where $P_{C_i}$ is the luminal capillary pressure of the $i$-th segment and $P_A$ is the alveolar pressure.

The pressure-flow relationship within the network is based on the computation of the resistance values of each segments. Once resistance values were computed, network hemodynamics, described by $n$ junction pressures ($P_{C_i}$) and $m$ segment flows($Q_i$), was computed by a linear analysis, which involved the solution of a $n \times m$ linear system. $m$ equations describe the relationship between pressure drop along a capillary, its flow $Q_i$ and its resistance $R_{seg_i}$:

$$Q_i = \frac{(P_{C2_i} - P_{C1_i})}{R_{seg_i}} \quad (Eq. 2.6)$$

where $P_{C1_i}$ and $P_{C2_i}$ are the luminal pressure at the junctions associated to the $i$ - th capillary segment.

$n - N_{BC}$ equations describe the equilibrium condition of mass conservation at each junction:

$$\sum_{j=1}^{m_s(i)} (Q_j - J_{v_j}) = 0 \quad (Eq. 2.7)$$

where $m_s(i)$ is the number of segments connected to the $i$ - th junction, $J_{v_j}$ is the filtration rate across the segment, depending on $P_C$. $N_{BC}$ are the boundary conditions, which can be either pressure or flow conditions. The linear system involves the construction of a matrix based on the equations 2.6 and 2.7 and on boundary conditions; by using standard methods (generalized minimal residual method), each junction pressure and each segment flow can be obtained.
Theoretical background of modelling lung perfusion and lung fluid balance

The computation of the resistance $R_{\text{seg}_i}$ of each capillary segment requires to include effects related to the two-phase nature of blood, which modifies fluid viscosity in narrow conduits.

**Non-linear effect in microcirculation**

Whilst large vessel hemodynamics can be described by Poiseuille’s equation assuming a constant viscosity with different vessel diameter, microcirculation is affected by non-linear mechanisms, named Fahraeus and Fahraeus-Lindqvist effects [71]. Fahraeus effect consists in the reduction of the hematocrit level as the vessel diameter is less than 300 $\mu$m; this phenomenon generates the so-called phase separation effect, that is a different distribution of hematocrit and thus blood flow between the daughter segments after a bifurcation. On the other side, Fahraeus-Lindqvist effect consists in a reduction of viscosity in narrower vessels; this effect can be explained by considering that the stream of red blood cells tend to accumulate in the core layer of the vessel, while plasma remains in the external one. This behaviour has been described by introducing the concept of apparent viscosity for non-Newtonian fluid, representing the equivalent viscosity of a Newtonian fluid having the same resistance. The apparent viscosity can be described by the following semi-empirical relationship [72]:

$$\mu_{\text{app}} = \frac{\mu_p}{\left[1 - \left(1 - \frac{\mu_p}{\mu_c}\right)\left(1 - 2 \times \left(\frac{D^*}{D_h}\right)^4\right)\right] \left(1 - \left(\frac{D_m}{D_h}\right)^4\right)}$$  \hspace{1cm} (Eq. 2.8)

$D_h$ is the capillary segment hydraulic diameter, equal to the diameter $D_i$ in case of cylindrical tubes. $\mu_p$ represents plasma viscosity assumed to be equal to 1.2 $cP$ (centipoises) [69], of cytoplasmic viscosity $\mu_c = e^{0.48+2.35\times H_d}$, where $H_d$ is the vessel hematocrit. $D_m$ is the diameter of the smallest vessel that a RBC can pass through ($2.7 \mu m$, [69]) and $D^* = 2.03 - 2 \times H_d [\mu m]$. If capillaries were assumed cylindrical, resistance values were computed for each segment $i$ by Poiseuille’s law:

$$R_{\text{seg}_i} = \frac{128 \times \mu_{\text{app}} \times L_i}{\pi \times D_i^4}$$  \hspace{1cm} (Eq. 2.9)

otherwise a complex integration involving Reynolds number and Darcy friction factor along segment length is required [68] [69].

Once all the resistance values are computed, a first estimation of flow and pressure can be obtained by applying Eq. 2.6 and 2.7 and considering boundary conditions, which can be pressure values at junctions or blood flow at segments. [69] considered also the presence of junctions into the computation of capillary resistance. A particular case is the computation of hemodynamics in large micro-vascular network having incomplete boundary conditions; a possible solution is to optimize blood flow so as to obtain reasonable shear stress experienced by capillary segments [73]. This constraint can be expressed by additional equation which introduces Lagrangian multipliers to variables and makes the whole system solvable by a linear equation system.
The first estimate of blood flow and pressure can be used to calibrate capillary vessels by considering the pressure-radius elastic relationship, provided by some experimental data [63]. Furthermore, due to the phase-separation effect, hematocrit depends on blood flow and thus need to be computed; a new analysis of segment capillary resistance is needed, due to the dependence of $R_{seg}$ to $H_d$, expressed by Eq. 2.8 and 2.9. This process is iterated until convergence of the hemodynamic solution.

The dependence of discharge hematocrit $H_d$ upon blood flow is estimated by the following model introduced by [74] and used by [69] and [75]. For each junction, being $Q_{input}$ the total blood flow entering in that junction and $Q_{output(i)}$ the blood flow rate in the $i$-th output segment of the junction, the ratio between the input volumetric flux of RBCs $Q_{RBC, input}$ and the output RBC flow in the $i$-th segment $Q_{RBC, output(i)}$ can be described by the following relationship:

$$\frac{Q_{RBC, output(i)}}{Q_{RBC, input}} = \begin{cases} 
G \left( \frac{Q_{output(i)}}{Q_{input}} \right) & \text{if } G \left( \frac{Q_{output(i)}}{Q_{input}} \right) \leq \frac{Q_{output(i)}}{Q_{RBC, input}} \\
\frac{Q_{output(i)}}{Q_{RBC, input}} & \text{if } G \left( \frac{Q_{output(i)}}{Q_{input}} \right) > \frac{Q_{output(i)}}{Q_{RBC, input}}
\end{cases} \quad (Eq. 2.10)$$

where $G$ is a sigmoidal function:

$$G \left( \frac{Q_{output(i)}}{Q_{input}} \right) = \begin{cases} 
0 & \text{if } 0 \leq \frac{Q_{output(i)}}{Q_{input}} \leq r \\
1 + \left( \frac{1 - \left( \frac{Q_{output(i)}}{Q_{input}} + r \right)^b}{\frac{Q_{output(i)}}{Q_{input}} - r} \right)^{-1} & \text{if } r \leq \frac{Q_{output(i)}}{Q_{input}} \leq 1 - r \\
1 & \text{if } 1 - r \leq \frac{Q_{output(i)}}{Q_{input}} \leq 1
\end{cases} \quad (Eq. 2.11)$$

where $r$ is the flux cutoff parameter, defining the minimal distributed fraction of RBCs to the daughter vessel: if $\frac{Q_{output(i)}}{Q_{input}} \leq r$, no RBC flow is considered to enter the daughter branch. Following [69] and [76], $r$ is assumed to be equal to 0.05. $b$ defines the sigmoidal shape of $G \left( \frac{Q_{output(i)}}{Q_{input}} \right)$ and is assumed equal to 1.15 [77]. Discharge hematocrit $H_d$ can be obtained by $H_{d(i)} = \frac{Q_{RBC, output(i)}}{Q_{output(i)}}$. 
2.3 Modelling of interstitial fluid balance

Fung model of interstitial fluid flow
In 1973, Fung proposed a model of interstitial fluid flow based on his "sheet-and-post" model [64]. "Posts" were identified as the sites of fluid accumulation and water is assumed to move across the blood-tissue barrier, following the revised Starling equation (Eq. 1.1). Fluid movement within interstitial tissue is described by the Darcy's law for porous media, stating that the velocity of fluid motion is proportional to the sum of the pressure gradient. To model the presence of a tissue pressure gradient throughout, Fung admitted the possibility of an interconnection between adjacent posts. Finally, tissue compliance $C$ is introduced to obtain tissue pressure values from increases of interstitial fluid volume value $(V - V_o)$:

$$P_{liq} = (V - V_o) / C \quad (Eq.2.12)$$

Results of the mathematical analysis showed that the interstitial water volume increases with pulmonary blood volume, independently from pressure values and lymphatic drainage, and correlates with arteriolar blood pressure. This pioneer theoretical work put the basis for understanding the complex the relationship between microcirculation and tissue fluid balance.

Electrical model
An electrical model of the pulmonary interstitial-lymphatic interaction was provided by [78] and depicted in Figure 2.1. Pulmonary interstitium is represented as a double capacitive compartment $C_1$ and $C_2$, where $C_1$ represents the capacitance of the tissue surrounding the end of lymphatic vessels, i.e. the alveolar septal interstitium and the small extra-alveolar blood vessels, whose tissue pressure $P_{liq}$ governs Starling equation (Eq. 1.1) for micro-vascular filtration $J_v$; $C_2$ represents peri-vascular and peri-bronchial capacitive compartment, where fluid accumulates if lymphatics are not able to remove septal interstitial excess fluid and thus including the tissue. Lymphatic flow $J_l$ is computed by considering that the pressure gradient across lymphatic depends on $P_{liq}$, the interstitial liquid pressure, on $P_{out}$, the pressure in the neck veins, where lymph flow is collected, and on $P_p$, the active pumping pressure of lymphatics:

$$J_l = \frac{P_{liq} + P_p - P_{out}}{R_L} \quad (Eq.2.13)$$

where $R_L$ is the lymphatic resistance.
Figure 2.1. Electrical model of the pulmonary interstitial-lymphatic system. Adapted from [78].

In [78], both $R_L$ and $P_p$ are supposed to be a function of interstitial fluid volume. In particular, $R_L$ depends on fluid volume in the peri-vascular compartment, due to mechanical stretching and compression, $P_p$ is proportional to interstitial fluid volume as was experimentally determined [79], and thus to $P_{\text{liq}}$. Another attempt of simulation was made by [80]; both fluid and protein fluxes are considered and simulated in time, by applying the Kedem and Katchalsky equations [81]. The first one is identical to Starling revised equation and describes fluid filtration, while the second one quantifies the protein flux $J_{sv}$ across the semi-permeable membrane of the capillary endothelial barrier:

$$ J_{sv} = J_v \cdot (1 - \sigma_f) \cdot \text{Conc}_c + (\text{Conc}_c - \text{Conc}_i) \cdot PS \quad (\text{Eq. 2.14}) $$

where PS is the product of the protein permeability $P$ and of exchange surface $S$ and $\sigma_f$ is the protein reflection coefficient. $\text{Conc}_c$ and $\text{Conc}_i$ represent the capillary and interstitial protein concentration, respectively. The first term of right member of Eq. 2.14 is the protein flux "dragged" by fluid flux, while the second term represent the diffusion contribution to protein flux, proportional to the concentration gradient. By comparing the experimental and simulated increased in interstitial volume, obtained by

$$ \frac{dV}{dt} = J_v - J_l \quad (\text{Eq. 2.15}) $$

and by Eq. 2.12, with different combinations of values for the protein permeability $P$, for the hydraulic conductivity $L_p$ and for $\sigma_f$, it was found that a lesional edema as those occurring after administration of oleic acid is mainly correlated to an increase in $L_p$ and subsequent increase in surface, while increases in $P$ and $\sigma_f$ were not sufficient to generate the observed lung weight gain.

A general model including all the driving forces involved in lung fluid balance was proposed by [32] and is represented in Figure 2.2.
Figure 2.2 Electrical model of the pulmonary capillary-interstitial-lymphatic system. Adapted from [32].

In [32], Eq. 1.1, 2.12, 2.13, 2.14 and 2.15 are used to build a 1-st order algebraic system describing the capillary-interstitial fluid and protein exchange and lymphatic drainage. By adopting the equilibrium criterium $J_v = J_l$, it is possible to compute the interstitial steady-state condition, expressed by $P_{liq}$ and $\text{Conc}_i$ [32], while solving the algebraic system, the temporal pattern of $P_{liq}, J_v$ and $J_l$ can be derived.
3. Computational micro-scale model of control of extra-vascular water and capillary perfusion in the air-blood barrier

The content of this chapter is based on the publication: Mazzuca E, Aliverti A, Miserocchi G. Computational micro-scale model of control of extravascular water and capillary perfusion in the air blood barrier, Journal of Theoretical Biology, vol 400, pp. 42-51, 2016.
3.1 Introduction

The control of the volume of extra-vascular water differs among organs and body compartments reflecting specific functional conditions. In the lung, which is by nature exposed to edemagenic conditions such as increased cardiac output and/or hypoxia, interstitial fluid at the level of the air blood barrier is kept at a minimum volume to favour gas diffusion [82] [6]. A key factor in the control of extra-vascular lung water is the very low compliance of lung parenchymal interstitium [83] that depends upon the macromolecular organization of the proteoglycans that act as link proteins within the interstitial matrix [84]. In fact, when an edemagenic condition develops ([85] [84] [86]), a minor increase in extra-vascular water causes a remarkable increase in liquid interstitial pressure $P_{liq}$ from a physiological value of about $-10 \text{cmH}_2\text{O}$, up to about $3 - 5 \text{cmH}_2\text{O}$ and this obviously buffers micro-vascular filtration [86]. A lumped-parameter model of control of extra-vascular lung water based on electrical equivalent circuit provided a framework for modelling the transition towards the development of lung edema, allowing to partition between hydraulic and increased permeability edema [32]. Little attention, however, has been devoted to the effect of the mechanical interaction between alveolar microcirculation and the increase in $P_{liq}$ due to fluid accumulation. A possible further potential mechanism preventing edema formation may be the so-called "Starling resistor effect", namely a decrease in alveolar perfusion due to the compressive effect of an increase in $P_{liq}$ [59].

The aim of this study was therefore to develop a morphologically-based non-linear model of alveolar-capillary unit (ACU), integrating fluid-dynamic equations of capillary flow and quantitative laws of interstitial fluid balance. The model considers the interaction between several variables impacting on micro-vascular fluid exchange, namely alveolar capillary density, perfusion of septal and corner vessels, blood flow resistance, $P_{liq}$ and micro-vascular permeability coefficients. Model simulations were compared with experimental data available in the literature.

3.2 Material and Methods

Figure 3.1 presents a schematic of the whole workflow for the characterization of the alveolar-capillary unit (ACU), composed by three steps: A) the construction of an anatomically-based ACU (ACU topology), B) the computation of ACU pressure-flow relationship (ACU perfusion) and C) dynamic simulations of ACU fluid exchanges by means of a 1st order algebraic system.
A. ACU topology

Figure 3.2A shows a model of lung alveolar sac, surrounded by a capillary network fed by terminal arterioles (TA, closed circles) and drained by collecting venules (CV, closed squares). CC and SC represent corner and septal capillary vessels, respectively. Based on this model, we defined an alveolar-capillary unit (ACU) as made of 20 contiguous alveoli [75], of 19000 µm² each, as reported for rabbit lung [63] (Figure 3.2B). For simplicity, the whole surface of each alveolus was given a squared shape, allowing to define a septal capillary network surrounded by corner vessels running at the boundary between adjacent alveoli. Grey and black vessels represent septal and corner capillaries, respectively. The capillary network is supplied by 4 TA, as in [75], and is drained by 5 CV [75].

To build the ACU, we used a 2D constrained Voronoi diagram. In the alveolar region considered, we chose a random distribution of $N_{Voronoi}$ points. In order to meet the morphological constraint on capillary diameter, we only accepted a minimal distance of 7µm between points. The remaining points represent the nodes around which the whole capillary network is drawn by “Voronoi tessellation”. $N_{Voronoi}$ was chosen so as to match the morphological constraint of the ratio between capillary and interstitial alveolar volume equal to 1.54, corresponding to the value found in the bottom of the lung (data from rabbits, [9]), thus reflecting the maximum extension of a pulmonary capillary network.

To compute alveolar and capillary surface and volume, we referred to the 3D geometrical model of septal capillaries and alveolar interstitial space, that is considered of the same thickness for the wall of the capillaries as well as for the portion of the intercapillary septum (Figure 3.2D). From [9], we assumed an average thickness for alveolar interstitial compartment of 1 µm (arithmetic mean thickness between the thick and thin portion of the air-blood barrier). Therefore, we calculated the alveolar surface area as $S_{alv} = 2 \times ACU\ area - 2 \sum_{i=1}^{m} r_i \times L_i + 2\pi \sum_{i=1}^{m} r_i \times L_i$, the capillary surface area as $S_{cap} = 2\pi \sum_{i=1}^{m} r_i \times L_i$, the interstitial volume as $V_{interst} = S_{alv} \times \tau$ and the capillary volume as $V_{cap} = \pi \sum_{i=1}^{m} r_i^2 \times L_i$. $r_i$ and $L_i$ are the radius value and length value of each capillary segment (Figure 3.2C), $m$ is the number of capillary segments.

B.1 Lung interstitial mechanics and capillary transmural pressure

Micro-vessel perfusion is critically dependent upon transmural pressure $P_{tm}$. For $P_{liq} < 0$, capillaries are strictly adherent to the surrounding structures and therefore, $P_{tm}$ can be assumed to be equal to $P_C$. With increasing $P_{liq}$ above atmospheric, due to fluid interstitial accumulation, $P_{liq}$ tends to compress capillaries resulting in flow limitation [59].
Figure 3.1 Workflow of the analysis of the ACU fluid-dynamic interaction between perfusion and filtration. First, an anatomically based geometric structure is created, then ACU perfusion is computed described in text and in Chapter 2. Finally, ACU micro-vascular fluid exchanges is computed. Reproduced from [87].

Since no data are available on the effect of interstitial pressure on capillary caliber, we assumed for simplicity that:

$$P_{tm} = \begin{cases} P_c & \text{if } P_{liq} < 0 \text{ cmH}_2\text{O} \\ P_c - P_{liq} & \text{if } P_{liq} > 0 \text{ cmH}_2\text{O} \end{cases} \quad (Eq.3.1)$$

In absence of vascular tone, the $P_{tm}$ versus capillary radius $r$ relationship was described by a linear equation, which fits data from [63]:

$$r[\mu m] = \frac{0.23 \times P_{tm}[cmH_2O] + 4.39[\mu m]}{2} \quad (Eq.3.2)$$

The coefficient $0.23 \mu m/cmH_2O$ is an estimate of capillary compliance $C_c$. 

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**Figure 3.2** A: model of lung alveolar sac, surrounded by a capillary network fed by four terminal arterioles (closed circles, TA) and drained by five collecting venules (closed squares, CV). CC: corner capillary vessels; SC: septal capillary vessels. B: top view of an alveolar-capillary unit (ACU), made of 20 contiguous alveoli, whose capillary network is supplied by four arterioles (closed circles) and drained by five collecting venules (closed squares). Grey and black vessels represent septal and corner capillaries, respectively. C: enlargement of a sub-region of ACU to identify septal and corner vessels and their length. D: 3D enlargement of the geometrical model of septal capillaries and alveolar interstitial space, whose thickness τ is assumed constant and equal to 1 µm. Capillary segment length $L_i$ varies depending on the distance between two successive bifurcations; capillary diameter $D_i$ depends upon transmural pressure and wall compliance. Reproduced from [87].

**B.2 ACU perfusion**

ACU pressure and flow distributions were computed by using the same approach of [69], described in details in Chapter 2. Initially, the same diameter value was assigned to all the capillaries, except corner vessels; this value is based on the pressure/radius relationship of Eq. 3.2, based on a pressure value corresponding to an average value of pressure between arteriolar and venular pressures. Corner vessels are capillary segments located at the boundary of the alveolar septal region [69]; since no data are available for corner vessels, we assumed their caliber to be 1.32 times the average diameter of a septal
capillary [69]. Then, linear analysis was performed to obtain pressure and flow values for each capillary segment in the network. Boundary conditions are required, namely arteriolar and venular pressures, \(P_{\text{t}q}\) and inlet hematocrit (set at 0.4, as in [69]). Solution implies an iterative approach (Figure 3.1), in order to consider non-linear effects. As in [69] and [75], asymmetrical hematocrit distribution at bifurcation, due to the Fahreus-Lindqvist effects, was considered. Hematocrit distribution influences capillary resistance (Eq. 2.8), so an iterative procedure was performed. Initially, capillary flows were calculated with initial values for hematocrit, equal to 0.4 in the whole network [69]. At each iteration, flow rates were used to calculate new capillary hematocrits, viscosities and resistances; furthermore, average pressures within each capillary segment were used to compute its radius, by using Eq. 3.2 and adding a \(\pm 5\%\) of random variability to account for physiological heterogeneity. Iteration was performed until convergence.

Once convergence is obtained, capillary segments having \(Q_{\text{RBC}} < 12 \text{ RBCs/s} [88]\) are considered unperfused and the network is reduced. In order to obtain the unit \(\text{RBCs/s} \text{ from m}^3/\text{s}\), it was assumed an average haemoglobin volume of 85 \(fL [89]\).

The index of recruiting \(\text{recr}\) describes the percentage of perfused capillary length (with respect to the whole initial capillary network) which thus contributes to micro-vascular fluid exchanges and to diffusion. The iterative procedure is then applied again to the reduced network until convergence. Inlet arteriolar pressure and venular outlet pressure (12 \(cmH_2O\) to 6 \(cmH_2O\), respectively) in control conditions were taken by in-vivo experimentally measured data in rabbits from [10] yielding a mean capillary pressure of about 9 \(cmH_2O\). In order to increase capillary perfusion, we only increased arteriolar pressure, which was found to be the only factor recruiting pulmonary capillaries (12, 32); venular pressure was assumed constant at 6 \(cmH_2O\). ACU perfusion was computed in three conditions: control \(P_{\text{t}q} = -10 \text{ cmH}_2\text{O} \), interstitial edema with \(P_{\text{t}q} = 1 \text{ cmH}_2\text{O} \) and with \(P_{\text{t}q} = 4 \text{ cmH}_2\text{O} \). The functions \(S_{\text{cap}} = S_{\text{cap}}(P_c, P_{\text{t}q}) \) and \(Q = Q(P_c, P_{\text{t}q}) \), obtained by interpolating perfusion results, were used in the dynamic simulations described in the following paragraph (Figure 3.1). Furthermore, a sensitivity analysis was performed to assess model robustness for ACU perfusion against the choice of the most relevant parameters, namely input hematocrit (\(H_d\)), capillary compliance (\(C_c\)), capillary density (\(N_{\text{Voronoi}}\)) and ACU dimensions (\(\text{size}\)). These features were varied each by \(\pm 10\%\) and the effects of these modifications on ACU input blood flow \(Q\) and ACU recruiting (\(\text{recr}\)) were evaluated.

\textbf{C.1 Dynamic simulations of the model}

Filtration is modelled by using the approach described in Chapter 2. We report here the same equations in order to introduce the chosen parameters and to justify them. The model of micro-vascular fluid and solute flux is based on the re-visitation of the classical Starling Equation, accounting for interstitial liquid pressure \(P_{\text{t}q}\):
\[
\frac{J_v}{S_{cap}} = L_p \left[ P_C - P_{liq} - \sigma_f (\pi_L - \pi_T) \right] \quad (Eq. 3.3)
\]
where \(\pi_L\) and \(\pi_T\) represent the plasmatic and interstitial colloidal osmotic pressure, respectively. \(L_p\) is the hydraulic conductivity and \(\sigma_f\) is the protein reflection coefficient set at 0.48, as in [32]. The pressure balance in brackets is the filtration Starling gradient. For each capillary segment, filtration flow towards interstitium was computed considering \(L_p = 1.3 \cdot 10^{-9} \text{m/s/cmH}_2\text{O}\) [34] and assuming its surface area equal to \(2\pi r_i \cdot L_i\). The whole ACU filtration flow was computed by summing \(J_v\) of only perfused capillaries.

Interstitial fluid balance is the result of the balance between the micro-vascular filtration towards lung interstitium \(J_v\) and the lymphatic drainage \(J_l\) [32]:

\[
J_l = \frac{P_{tiq} + P_p - P_{out}}{R_L} \quad (Eq. 3.4)
\]

where \(P_p\) is lymphatic pumping pressure (assumed equal to 27 cmH\(_2\)O, [32]), \(P_{out}\) is lymphatic outlet pressure (equal to 3 cmH\(_2\)O [32]) and \(R_L\) is lymphatic resistance. \(R_L\) value was computed so as to match \(J_v\) when \(P_{tiq} = -10\ cmH_2O\) (equilibrium condition, \(J_v = J_l\), for mass conservation). When a perturbation alters equilibrium conditions, dynamics can be computed by equalling the rate of change of fluid interstitial volume \(V\) to the difference between interstitial inflow (\(J_v\)) and outflow (\(J_l\)):

\[
\frac{dV}{dt} = J_v - J_l \quad (Eq. 3.5)
\]

Interstitial compliance \(C = \frac{1}{V_0 \frac{dV}{dP_{tiq}}}\) was considered constant and equal to 0.004 ml \cdot ml\(^{-1}\) \cdot cmH\(_2\)O\(^{-1}\) [85]. \(V\) is the interstitial fluid volume and \(V_0\) is considered equal to the fluid interstitial volume in control condition (\(P_C = 9\ cmH_2O, P_{tiq} = -10\ cmH_2O\)).

To evaluate the effect of large perturbation in ACU fluid balance, we simulated two different extreme conditions: 1) a remarkable (8-fold) increase in \(L_p\), reflecting the occurrence of an acute inflammation (31) simulating the so-called permeability-induced edemagenic condition, 2) a remarkable increase in \(P_C\) (from 9 up to 25 cmH\(_2\)O), simulating a hydraulic-induced lung edema, as reported to occur on increase in cardiac output (heavy exercise) [48].

C.2 Comparison between simulated and experimental data from literature

We attempted to fit the time course of \(P_{tiq}\) experimentally measured in two different edemagenic protocols, namely acute hypoxia exposure to 12% oxygen [86] and collagenase injection [10]. These two protocols were similar in that they led to the same peak positive value of \(P_{tiq}\), during initial edema formation, averaging about 4 cmH\(_2\)O, a condition defined as interstitial edema. Time to reach peak \(P_{tiq}\) values were 360 minutes for hypoxia exposure and 40 minutes for collagenase injection.

Fitting \(P_{tiq}\) experimental data allowed to estimate, for the different protocols, the time dependent changes in coefficients and pressure appearing in the Starling equation.
causing the increase in micro-vascular filtration, namely: 1) the hydraulic conductivity \( L_p \), 2) protein reflection coefficient \( \sigma_f \) and 3) the capillary pressure \( P_C \) (that in turns affects filtration surface through capillary perfusion). To fit the data, we modeled changes in \( L_p \), \( \sigma_f \) and \( P_C \) considering either a linear or exponential \( (Y = Y_{max} \cdot (1 - e^{-\frac{t}{\tau}})) \) increase in time, where \( \tau \) is the time constant.

For hypoxia, oncotic gradient was assumed to be constant and equal to 7.4 \( cmH_2O \) [90]. Relative to collagenase injection model, protein exchange was considered. We computed both plasma and interstitial oncotic pressure \( \pi \) assuming a linear relationship with protein concentrations, given by \( \pi[cmH_2O] = 0.5 \cdot Conc[kg/m^3] \), obtained by linearizing the quadratic relationship found in [85].

As in [32], protein flux from plasma to interstitial compartment \( (J_{sv}) \) was computed by Eq. 2.14:

\[
J_{sv} = J_p \cdot (1 - \sigma_f) \cdot Conc_c + (Conc_c - Conc_i) \cdot PS \quad (Eq. 3.6)
\]

where PS is the surface area-protein permeability product, \( Conc_c \) and \( Conc_i \) are plasmatic and interstitial protein concentrations, respectively. As in (5), lymphatic protein drainage \( J_{sl} \) is given by:

\[
J_{sl} = Conc_i \cdot J_l \quad (Eq. 3.7)
\]

Mass conservation for interstitial proteins implies

\[
\frac{dQ_p}{dt} = J_{sv} - J_{sl} \quad (Eq. 3.8)
\]

where \( Q_p \) is the interstitial protein content and is related to \( Conc_i \) by the relationship \( Conc_i = Q_p / V \).

### 3.3 Results

**ACU perfusion**

Figure 3.3A shows that ACU recruitment increases with increasing \( P_C \) and this relationship is displaced progressively downward on increasing \( P_{liq} \). This de-recruitment of the septal network is reflected also by the decrease in ACU flow, of filtration surface and of filtration flow on increasing \( P_{liq} \) (Figure 3.3, Panels B,C and D, respectively). Filtration flow is also buffered by a decreased hydraulic gradient. Color panels of Figure 3.4 reports the capillary perfusion patterns using a color code for the ACU on varying \( P_C \) and \( P_{liq} \). The top side of the figure reports the calculated ratio of septal to corner flow at increasing distance from the arteriolar to the venular end of the ACU. For \( P_C = 9 \ cmH_2O \) and \( P_{liq} = -10 \ cmH_2O \), essentially most of the control ACU flow is equally distributed between septal and corner vessels; however, with increasing \( P_{liq} \) septal flow is drastically reduced. For \( P_C = 14.5 \ cmH_2O \), septal flow further increases relative to corner flow, irrespective of \( P_{liq} \).
Sensitivity analysis

Table 3.1 shows the results of the sensitivity analysis. Capillary compliance was found to be the most effective parameter, producing a 15% variation of $Q$, greater than that found by ([69], [75]). The reason for the difference relies on our assumption of capillary circularity, different from the semi-circular shape proposed by ([91],[75]). Effects of capillary density and hematocrit are negligible, while ACU size is almost linearly correlated with ACU resistance and thus with $Q$ and $re_{cr}$.

![Graphs showing sensitivity analysis results](image)

**Figure 3.3** Results of ACU perfusion analysis, obtained by varying inlet ACU pressure, and thus mean capillary pressure ($P_C$), and interstitial pressure ($P_{liq}$). A: recruitment patterns of ACU, expressed as a percentage of recruited capillary length in the network ($re_{cr}$), in control conditions ($P_{liq} = -10 \text{ cmH}_2\text{O}$, full dots) and during interstitial edema ($P_{liq} = 1 \text{ cmH}_2\text{O}$, empty triangles and $P_{liq} = 4 \text{ cmH}_2\text{O}$, closed squares). B: ACU input flow (same conditions as Panel A). C: ACU filtration surface $S_{cap}$ (same conditions as Panel A). D: ACU filtration flow $J_v$ (same conditions as Panel A). Modified from [87].

Dynamic simulations

Figure 3.5 shows the dynamic simulations for two different perturbations of interstitial fluid equilibrium. Blue lines refer to an 8-fold increase in $L_p$, exponential ($tau= 4$ minutes, solid line) or linear (dashed line). Red lines refer to an increase in $P_C$, either exponential ($tau= 4$ minutes, solid line) or linear (dashed line), from 9 to 25 $\text{ cmH}_2\text{O}$. Simulation results show that micro-vascular filtration and lymphatic drainage (undistinguishable) almost asymptotically double in all four cases, with a greater
increase for $P_c$ perturbation. In the case of permeability-induced edema, the resulting increase in $P_{liq}$, that however levels slightly above 0 cm$H_2O$, decreases remarkably the Starling filtration gradient, but decreases by only about 27% ACU blood flow and by 30% filtration surface. In the case of hydraulic-induced edema, the resulting increase in $P_{liq}$, that reaches about 4 cm$H_2O$, maintains a high Starling filtration gradient, and an almost doubled filtration surface due to capillary recruitment with a 5-fold increased ACU blood flow.

**Comparison between simulated and experimental data from literature**

The case of hypoxia exposure: Top left panel of Figure 3.6 shows the increase in $P_{liq}$ in an animal experimental model exposed to 12% O$_2$ up to six hours [86]. Increasing linearly micro-vascular hydraulic conductivity $L_p$ (2-fold, case a, solid line) over time (360 minutes) did not fit experimental data. A similar result was obtained for an increase in capillary pressure (up to 17 cm$H_2O$, case b, dotted line), even considering the $P_c$-dependent increase in filtration surface. Combining the linear increase in $L_p$ and $P_c$ allowed a very good fitting (case c, dashed line). Figure 3.6, bottom left panel, shows that, for case c, ACU flow attains a 4.5-fold increase. The close matching between the increase in micro-vascular filtration and in lymphatic drainage was preserved, although the latter was delayed by an unappreciable time.

The case of collagenase injection: Top right panel of Figure 3.6 shows experimental data of the increase in $P_{liq}$ following collagenase injection [5]. By only a 8-fold exponential increase (time constant of 17 minutes) of $L_p$ (case a, solid line), the model did not adequately fit the data up to 30 minutes, while this could be obtained by including an exponential halving of $\sigma_f$ (time constant of 17 minutes) (case b, dotted line). The subsequent increase in $P_{liq}$ could be modeled by a small linear increase in $P_c$ from 9 to 11 cm$H_2O$ (case c, dashed line).

Bottom right panel of Figure 3.6, referring to case c, shows that the steep increase in $P_{liq}$, reflecting the parallel increase in filtration rate and lymphatic flow, causes a reduction in ACU flow, that lasts from about 12 up to 45 minutes, when the increase in $P_c$ is sufficient to recruit alveolar capillaries, restoring a value of ACU blood flow close to baseline, before de-recruiting occurs again.
Figure 3.4 Color panels show capillary recruitment of ACU at different $P_{\text{liq}}$ and $P_{C}$, with color-coded log-scale intensity of RBC flows. On the top, septal to corner capillary flow ratio along ACU length; solid line for $P_{\text{liq}} = -10 \text{ cmH}_2\text{O}$, gray line for $P_{\text{liq}} = 1 \text{ cmH}_2\text{O}$ and dark gray line for $P_{\text{liq}} = 4 \text{ cmH}_2\text{O}$. Reproduced from [87].
Table 3.1 Sensitivity analysis of parameters. Effects of variation of input hematocrit, capillary compliance, ACU capillary density and ACU size on ACU input flow, ACU recruiting and average transit time of red blood cells. Control conditions are $P_C = 9 \text{ cmH}_2\text{O}$ and $P_{liq} = -10 \text{ cmH}_2\text{O}$. * The initial number of Voronoi points was varied without varying ACU size. ** ACU size was varied by varying also $N_{Voronoi}$ in order to achieve the same capillary density.

![Dynamic simulations of the model. Two edemagenic conditions are considered, providing four input time patterns: a 8-fold increase in $L_p$ (blue lines) and an increase in $P_c$ from 9 to 25 cmH$_2$O (red lines), either exponential (tau=4 minutes, solid line) or linear (dashed line). The output variables of each of the four perturbation are: filtration and drainage, normalized to baseline (their difference cannot be detected); $P_{liq}$; Starling filtration gradient; filtration surface; ACU blood flow. Adapted from [87].](image)
Figure 3.6 Comparison with experimental data. Left top: Fitting of experimental data of increase in $P_{liq}$ in rabbits on hypoxia exposure [86] (closed dots), a condition implying both an increase in $L_p$ and $P_C$ as specified in the legend. Left bottom: ACU perfusion, filtration and lymphatic flows in the best fitting case c. Right top: Fitting of experimental data of increase in $P_{liq}$ in rabbits following collagenase injection [5] (closed dots) a lesional condition implying an increase in $L_p$, $P_C$ and a decrease of $\sigma_f$ as specified in the legend. Note that the linear increase in $P_C$ starts at 30 minutes. Right bottom: ACU perfusion, filtration and lymphatic flows in the best fitting case c. Adapted from [87].

3.4 Discussion

In the present study, an anatomically-based model is proposed to describe the control of extra-vascular lung water and capillary flow based on the mechanical interaction between pressures generating trans-vascular flows, when the lung is exposed to edemagenic conditions. The model describes the phase defined as “interstitial edema”, characterized by an increase in $P_{liq}$ up to about 4 cmH$_2$O, which is considered as an important protecting factor against the development of severe edema [6].
The constitutive equations (Figure 3.1) are based on recognized model governing microvascular fluid exchanges [32], but extend it by including the mechanical dependence of capillary flow on pressures developing in the tissue due to edemagenic conditions. This required the definition of a morphologically-based capillary network and the computation of ACU perfusion.

**ACU topology**

The ACU network is based on data from rabbits, both for alveolar surface [92] and for volume capillary density per unit of interstitial volume [9]. This structural parameter relates the filtering capillary surface to the capacitance of the compartment receiving filtered fluid. In the topological model shown in Figure 3.2B, the ratio of corner vessel length/alveolar surface is $552 \mu m / 19000 \mu m^2 = 0.029 \mu m^{-1}$. Note that assuming a dodecahedral model for alveolar shape would provide a ratio of $900 \mu m / 19000 \mu m^2 = 0.047 \mu m^{-1}$. Therefore, compared to the dodecahedral model, we underestimate corner capillaries overall length and thus corner flow of about 35%. With a dodecahedral model, septal capillary de-recruitment due to increase in $P_{liq}$ would even be larger than that shown in Figure 3.3. At present, no data are available about the partition between septal and corner blood flow in edemagenic condition.

**Micro-vascular perfusion and fluid exchanges**

In control condition, with a pre-capillary arteriolar pressure of 12 cmH$_2$O and a post-capillary venular pressure of 6 cmH$_2$O, the flow per ACU unit amounts to $4.2 \times 10^{-5} \text{ mL/min}$; considering a total of $135 \times 10^6$ alveoli [92], the overall cardiac output would amount to $283 \text{ mL/min}$, a value compatible with data reported in unanesthetized rabbits [93].

In control condition, capillary recruitment shows a sharp increase above 8 cmH$_2$O, which is consistent with experimental data in intact thorax in rabbits [94]. Alveolar blood flow is sensitive to the increase of $P_{liq}$ above 0 cmH$_2$O. For a $P_C$ of about 9 cmH$_2$O, an increase in $P_{liq}$ up to 1 cmH$_2$O causes a minor capillary de-recruitment. A further increase of $P_{liq}$ up to 4 cmH$_2$O causes a remarkable de-recruitment (Figure 3.3A), as a consequence of a decrease in capillary transmural pressure and a corresponding decrease in ACU input flow (Figure 3.3). The de-recruitment of the septal circulation implies a progressive contribution of corner vessel to ACU blood flow (Figure 3.4). This "blood flow preserving" mechanism by corner vessels was found also in in-vivo experimental conditions [13], when alveolar pressure was raised above pulmonary arterial pressure. Furthermore, as shown in Figure 3.3A, derecruited capillaries due to $P_{liq}$ reaching 4 cmH$_2$O can be re-perfused by a small increase in $P_C$ of the order of 3 cmH$_2$O which can be achieved either through a precapillary vasodilation and/or by an increase in arterial pressure.

**Dynamic simulations**

Figure 3.5 shows that during the development of interstitial edema, filtration is almost doubled and is matched by a similar increase in lymphatic drainage. Therefore, as long
as the compliance of the matrix remains low, lung interstitium is well-designed to resist interstitial edema formation since lymphatics are able to remove interstitial excess filtrate. The presence of small terminal lymphatics has been questioned due to the anatomical constraint imposed by the thinness of the air–blood barrier [6], but small lymphatics were found in proximity of the alveolar vessels [95]; moreover, a recent histological study has reported the presence of one terminal lymphatic micro-vessel every two terminal arterioles in a human normal lung [96].

Eq. 3.1 represents the basis for the so-called "Starling resistor effect", consisting in an increase of vascular resistance due the compression exerted of external pressure, in our case related to an increased interstitial pressure. The hypothesis of a compressive effect of elevated interstitial pressure has been put forward by several investigators, regarding a wide range of disease conditions, from tumours [60] to ischemic tissues [59]. The compressive effect of lung edema has been recently hypothesized by [97] for a model of lung injury (oleic acid), where exogenous agents increasing cardiac output worsened the condition of microvascular filtration, maybe due to the reopening of capillaries previously collapsed due to lung edema.

Due to the assumption of Eq. 3.1, the functional control system of extra-vascular lung water does not essentially interfere with lung tissue perfusion, as long as \( P_c \) remains constant at control value (Figure 3.5). In fact, the increase in \( P_c \) generates a greater perturbation in lung fluid balance, being highly edemagenic due to the increase of both the Starling gradient and of the capillary surface available for filtration.

In general, the two conditions described by Figure 3.5 are variously combined within the whole lung after edemagenic perturbations and are also affected by active vasomotor responses of pulmonary vessels, not included in the model. Available experimental data confirm the heterogeneity in both regional lung fluid accumulation and perfusion in edemagenic conditions. Data in humans confirmed blood flow limitation in edemagenic lung regions, despite the administration of a vasodilator agent [98] and furthermore, redirection of blood flow from edematous to normal lung regions was correlated to corresponding arteriolar vasoconstriction and vasodilation, respectively [99]. Possibly, differences in capillary density may affect blood flow redistribution and thus regional interstitial fluid balance [9] and can be included in a organ-oriented model able to capture regional differences of edema development.

**Comparison between simulated and experimental data from literature**

Attempting to fit the experimental data of \( P_{\text{litq}} \) in models of interstitial edema allows to provide indications concerning the time evolution of key parameters affecting micro-vascular filtration, thus describing the functional adaptive response to an edemagenic condition.

**The case of hypoxia exposure (12% O\(_2\))**

Hypoxia exposure represents a characteristic edemagenic condition due to an increase in \( L_p \) and in capillary pressure and perfusion [85]. As shown in Figure 3.6, top left panel,
the best fitting was obtained by coupling a linear increase of \( L_p \) (2-fold, an increase compatible with that found in [100]) with an increase in \( P_C \) from 9 to 17 cmH\(_2\)O (compatible with the increase in pulmonary arterial pressure measured in [85]). Considering that the adaptive response to hypoxia is aimed to sustain oxygen delivery to the tissue, the model suggests that this can be achieved only through an increase in \( P_C \) that allows a 4.5-fold increase in ACU blood flow. This is achieved despite the compressive effect on capillary patency due the increase in \( P_{liq} \) from 200 minutes onwards. One may question if hypoxia exposure may affect blood hematocrit and thus ACU perfusion. It has been reported that variation in hematocrit due to lethal acute hypoxia does not exceed 5% [101]; since a \( \pm 10\% \) variation of hematocrit does not significantly influence micro-vascular perfusion and thus filtration of the model (Table 3.1), this effect can be neglected in the model.

**The case of collagenase injection**

Collagenase injection generates a considerable damage to capillary barrier thus increasing remarkably filtration rate within a short time [5]. The dynamical modelling considered only the interstitial phase of edema, characterized by an increase of \( P_{liq} \) up to 4 cmH\(_2\)O; Figure 3.6, right top panel, ignores the second phase of decrease of \( P_{liq} \) to 0 cmH\(_2\)O, as reported by [5], where the structural components are rapidly degraded and alveolar edema occurs. Best fitting of data up to 30 minutes was obtained with an 8-fold exponential increase in \( L_p \) and a halving of \( \sigma_f \), both with a time constant of 17 minutes. To fit the last 20 minutes, a small increase of \( P_C \) from 9 to 11 cmH\(_2\)O was hypothesized; this could be explained by either an increase in ACU resistance due to capillary compression or by an increased cardiac output, necessary to re-perfuse the derecruited ACU. The increase in \( L_p \) is comparable with the one reported by [100] in a model of hypoxia-reperfusion that also implies a remarkable increase in \( L_p \). In this case, considering Figure 3.3, the early event due to large increase in filtration rate implies shifting from top left to top right, moving then downward according to the increase in \( P_C \) to reestablish baseline blood flow. Also in this case, due to the increase in \( P_{liq} \) as long as interstitial compliance is kept low, the increase in filtration and lymphatic flows is less than 2-fold and their matching is preserved.

**Strengths, limitations and future developments of the model**

The model elucidates how the increase in \( P_{liq} \), reflecting the in-born low compliance of the lung interstitial matrix, represents a passive efficient mean to control extra-vascular water in ACU at risk of developing severe edema by limiting micro-vascular filtration and blood flow.

At the same time, the model explores the consequences of the mechanical interaction between interstitial and capillary pressure showing how, in edemagenic conditions, capillary blood flow can be maintained or even increased to assure oxygen delivery by limiting at the same time micro-vascular filtration.

This model has been developed for a single saccular structure, but it cannot be assumed
that the interaction among parameters involved in the model are similar in all lung ACUs. In fact, on comparing different lung regions, a considerable heterogeneity has been reported in the experimental model concerning the resistance to develop [99], suggesting a wide range of variability concerning the interaction of all the factors affecting micro-vascular fluid exchanges. The model should be also extended by integrating the active response (vasodilation/vasoconstriction) of pulmonary pre- and post-capillary micro-circulation in interstitial edema.

Concerning the role of non-perfused capillaries to micro-vascular fluid exchanges, we assumed that they do not contribute to filtration. However, data from [102] suggest that non-perfused capillaries still contribute to fluid exchange, being retrogradely filled through the existing network of interconnecting capillaries. An alternative hypothesis proposed that a cyclic change in capillary perfusion occurs and non-perfused segments provide fluid reabsorption from the interstitial compartment due to a remarkable decrease in $P_c$ [103].

Extension to human lung of the model could be envisaged by adapting ACU morphology to match alveolar size as provided by [92] and capillary surface density by [4]. Furthermore, the gravity-dependent effect should be included by considering regional variations in lung perfusion.

Several assumptions were made for dynamical modelling. First, a low coefficient of reflection $\sigma_f$ (ranging around 0.8 for dogs [85]) was assumed; a higher value would have an increased protective role: it would decrease both the Starling gradient of Eq. 3.3 and the protein flow (Eq. 3.6) and thus increase the oncotic gradient across the endothelial barrier. A sensitivity analysis is required to evaluate the effect of the choice of $\sigma_f$. Furthermore, lymphatic flow increase could be limited to a physiological value, over which drainage is not able to buffer further microvascular filtration. Finally, a complete compartmental model should include also the alveolar region; severe or alveolar edema occurs due to the structural degradation of the interstitial space, related to the partial fragmentation of the proteoglycan matrix [5] and causing a decrease of the interstitial compliance $C$. In this case, an additional flow towards the alveolar compartment and a variable interstitial compliance should be included in the model. Anyway, in the conditions modelled in Figure 3.6, the assumption of a constant compliance $C$ seems reasonable, at least in the initial phase of interstitial edema, during which $P_{\text{tuq}}$ can increase up to 4 cmH2O, due to the low fluid compliance of the interstitial matrix.

Despite these limitations, this study offers the tool to model several conditions of perturbation of lung fluid balance implying a corresponding modification of the mechanical interaction between capillaries and interstitial tissue. Several cases could be considered ranging from extreme physiological conditions, such as exhaustive exercise at extreme altitude to clinical conditions. Among the latter one can recall: a) passive mechanical lung ventilation, known to greatly affect micro-vascular lung perfusion, b) adult and neonatal acute respiratory distress syndromes, and last but not least, the recovery from severe edema.
4.5 Conclusions

1) This study presents a comprehensive model of the pulmonary alveolar capillary unit integrating several pulmonary capillary perfusion and lung fluid balance.
2) Applying the model to fit experimental data of interstitial liquid pressure measured in edemagenic condition allows to estimate the main patho-physiological mechanisms underlying edema development.
3) The model predicts an increase of blood arteriolar pressure possibly related to either an increase of cardiac output for hypoxia exposure or to the compressive effect of interstitial pressure on capillaries.
4) The model can be extended and applied to morphology of different species.

Appendix. Extension to human lung

Extension of the model to human lung can be accomplished by adapting morphological properties to data found in stereological evaluation of human lung. ACU size was chosen so as to have a mean alveolar surface of 120000 µm², a value found through alveolar reconstruction by serial sections for human lung [92]. The density of micro-vascular capillary can be based only on data on animals, since but no information is present in literature for human lung. In particular, the choice of the number of Voronoi points used to draw the capillary network can be based on the following morphological constraints: 1) the ratio between the capillary surface and the alveolar surface equal to 0.86 (data from rat, [104]), 2) the ratio between capillary and interstitial alveolar volume equal to 1.54, corresponding to the value found in the bottom of the lung (data from rabbits, [9]). Based on this data, the number of Voronoi points can be found to match the previous constraints. Figure 3.7A shows the ratio between the increase of capillary and alveolar surface with increasing Voronoi points; for the value provided by morphometric estimates of 0.86, the corresponding Voronoi number is 18000. Figure 3.7B shows that for a Voronoi number of 18000, the ratio between capillary and interstitial alveolar volume is about 1.52, close to the value reported by [9] for the more perfused part of the lung at functional residual capacity. Figure 3.8 represents the increase of capillary recruitment in case of a human network shows that ACU recruitment increases with increasing $P_C$ and this relationship is displaced progressively downward on increasing $P_{liq}$. This de-recruitment of the septal network is reflected also by the decrease in ACU flow on increasing $P_{liq}$ (Figure 3.8B). Figure 3.8C shows that ACU flow resistances increase remarkably on decreasing $P_C$ and increasing $P_{liq}$. Red blood cells transit times, shown in Figure 3.8D, were simply estimated by dividing the whole ACU capillary
volume by the ACU input flow and normalizing this value by 1.4, which is the ratio of RBC transit time to the plasma transit time [69]. Figure 3.8D shows that the average transit time decreases on increasing $P_C$ and decreasing $P_{\text{liqu}}$.

**Figure 3.7** Morphometric parameters of the human ACU as a function of the initial Voronoi points. **A**: capillary to alveolar surface ratio, compared with the reference provided by [104] (dashed line). **B**: capillary to interstitial volume ratio, compared with the reference provided by [9] (dashed line for both the dependent and non-dependent regions of the lung).
Figure 3.8 Main hemodynamic parameters for human ACU (capillary recruitment, Panel A, ACU input flow, Panel B, ACU resistance, Panel C, Red blood cell transit time, Panel D) as a function of mean capillary pressure.

With respect to the network adapted to rabbit size, it should be noted the higher perfusion pressure level at which recruitment occur and also the higher ACU blood flow, which, multiplied by the 500 million alveoli per lung [105] provides an estimate close to 5L/min. Blood capillary transit time obviously bears an inverse relationship with mean $P_C$ (Figure 3.8D), with values similar to those experimentally found in [106]. Figure 3.9 reports the capillary perfusion patterns using a color code for the ACU on varying $P_C$ and $P_{liq}$. The top side of the figure reports the calculated ratio of septal to corner flow at increasing distance from the arteriolar to the venular end of the ACU. For the lowest perfusion pressure (10.5 cmH$_2$O), essentially most of the control ACU flow is equally distributed between septal and corner vessels; however, with increasing $P_T$ septal flow is drastically reduced. With increasing $P_C$ (12 cmH$_2$O), the septal flow becomes 4 times larger relative to the corner flow for $P_{liq} = -10$ cmH$_2$O; yet, increasing $P_{liq}$ up to 3 cmH$_2$O again remarkably decreases septal flow. For the highest $P_C$ values, septal flow further increases relative to corner flow, irrespective of $P_{liq}$. With respect to rabbit ACU,
septal to corner flow ratio values reach higher values, due to higher capillary density and extension. The scalability of the approach makes possible to investigate the allometry of pulmonary capillary network throughout different species.

**Figure 3.9** Color panels show capillary recruitment of human ACU at different $P_{liq}$ and $P_C$, with color-coded log-scale intensity of RBC flows. On the top, septal to corner capillary flow ratio along ACU length; solid line for $P_{liq} = -10 \text{ cmH}_2\text{O}$, gray line for $P_{liq} = 1 \text{ cmH}_2\text{O}$ and dark gray line for $P_{liq} = 3 \text{ cmH}_2\text{O}$. 
4. From morphological heterogeneity at alveolar level to the overall mechanical lung behavior: an in vivo microscopic imaging study

The content of this chapter is based on the following publications:

E. Mazzuca; C. Salito, I. Rivolta, A. Aliverti, G. Miserocchi "From morphological heterogeneity at alveolar level to the overall mechanical lung behavior: an in vivo microscopic imaging study." Physiological Reports. 2014 Feb 7;2(2):e00221;

4.1 Introduction

Lung parenchyma is considered as a system composed by polyhedral alveoli opening on the lumen of alveolar ducts [107]. The tissue structure surrounding alveoli is a complex interconnected network of macromolecules with viscoelastic and elastic properties. Alveoli also present a liquid interface with air implying the existence of surface forces. Essentially, whole mechanical lung behavior reflects the mechanical properties of single alveoli whose distending pressure is given by the sum of an elastic component, developed by the parenchymal structures, and a pressure component reflecting surface forces. Several studies have considered how considerable perturbation of physiological condition at lung periphery affect the intrinsic viscoelastic properties and/or surface forces creating regional heterogeneities in the mechanical behavior. Conditions studied include remarkable decrease of airways caliber following bronchoconstriction [108], chemical destruction of the elastic component [109], and acute lung injury [110].

A recent article by Wilson [111] raised the question about regional differences in parenchymal microstructures as the basis for the variance in regional specific ventilation in physiological condition. In particular, Wilson [111] proposed that, in physiological conditions, regional differences in volume oscillations may actually result from the complex interaction between the stiffness of the elastic elements of the alveoli and surface tension relaxation.

We wished to provide a contribution to this hypothesis, addressing specifically two related questions: a) which are the differences in absolute and specific compliance: the former is defined as the change in volume over change in distending pressure, while the latter is defined as the absolute compliance over initial alveolar volume and represents the intrinsic mechanical property of the elastic components; b) how the potential heterogeneity in alveolar mechanical properties impacts on whole lung mechanics. To this aim, we developed an experimental method that allowed us to image subpleural airway terminal units keeping pleural space intact; this approach allowed a clear and neat view of unrestrained subpleural structures exposed to changing physiological local distending pressure. We estimated alveolar compliance under static conditions, thus neglecting a phase shift between elastic and surface forces, as hypothesized by Wilson [111].

4.2 Materials and Methods

Animal preparation.
A general consensus for the experimental procedures used in our research activity was obtained from Milano-Bicocca University Ethical Committee. Experiments were
performed on 6 adult (New Zealand White) male rabbits (weight range 1-1.5 kg) anesthetized with a bolus of 2.5 ml/kg of a saline solution containing 0.25 g/ml of urethane plus 10 mg/ml of pentobarbital sodium injected into an ear vein. The absence of an eyelid closure reflex was used as an estimate of the proper level of anesthesia during the experiment. When necessary, subsequent doses of 0.5 ml of anesthetic were administered through a venous line. Tracheostomy was performed, and animals were intubated. Before connecting the rabbit to the ventilator, paralysis was accomplished by pancuronium bromide (1 mg/kg body weight initial dose, supplemented by 0.33 mg/kg every 40 min). Mechanical ventilation was provided with a tidal volume (VT) of ~20 ml and a frequency of 16 breaths/min. The inspiratory flow signal was measured by a Fleisch-type pneumotachograph (Sensym, Milpitas, CA) and integrated in order to obtain lung volume; volume drift was avoided by correcting flow offset. Tracheal and esophageal (through esophageal balloon) pressures were measured by pressure transducers. Lung volume values were normalized to their maximum value, assumed at 20 cmH₂O.

**In vivo microscopy**
A typical in-vivo image is shown in Figure 4.1, where the alveolar texture is clearly visible. The “intact pleural window” was prepared in the sixth intercostal space to allow a view of the lower lobe where the cardiac artefacts are relatively small. The skin and superficial muscles on the right side of the chest were resected and, when reaching the layer of the intercostal muscles (indicated by “b” in Figure 4.3A), a surface area of about 0.5 cm² was freed from muscles down to the endothoracic fascia. Under stereomicroscopic view and with fine forceps, the endothoracic fascia was then carefully stripped, exposing the transparent parietal pleura (about 10 μm thick) through which freely moving subpleural alveolar structures could be neatly visualized (area indicated by “c” in Figure 4.3A). The microscopic image in Figure 4.3B highlights the difference between the stripped area (indicated by ”c1”) and the un-stripped one (indicated by “c2”).

**Labview protocol for end-expiratory triggering**
Image acquisition was implemented in Labview® environment. The main task the software fulfills is to allow the synchronization of image acquisition with a certain signal of respiratory mechanics. Mechanical ventilation is monitored by acquiring flow signal at a proximal position with respect to the tracheal aperture is subject to artefacts related to the cardiac movement and to the residual activity of respiratory muscles. The half-wave conformation of a half wave during the inspiratory cycle (Figure 4.2B) allows to identify the inspiratory peak, by looking for a minimum by an adaptive minimum research. Once detected the peak inspiration, the acquisition of three images at a frame rate of 10 Hz is triggered during the expiratory plateau phase, after a period equal to 0.6 times the previously estimated respiratory period $T_{resp}$. 

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**Figure 4.1** In-vivo microscopic imaging of a subpleural region. Alveolar structures are defined by a white border, while septal interstitium contains both corner vessels and interstitial components (epithelial and endothelial cells, extracellular matrix). Small vessels running on the subpleural region distribute blood flow to the capillary network. Reprinted with permission from [112].

**Figure 4.2** Experimental setup. **A:** Ring illuminator on thoracic surface and subpleural window. **B:** Typical pattern of flow measured at the inspiratory output of the mechanical ventilator. Temporal parameters evaluated for image acquisition triggering.

**Experimental protocol. Image acquisition.**
The animal was placed in left lateral decubitus under the field of a fixed microscope (Nikon's Eclipse FN1) connected to a video camera (CoolSNAP EZ, Photometrics) which
was interfaced through a IEEE-1394 data-transfer interface card with a personal computer equipped with an image-processing software (MetaMorph® System, Molecular Devices). Total microscopic magnification was 60x. A LED ring-light illuminator was anchored to microscopic optics to provide a uniform lighting of the alveolar field (Figure 4.2A). The depth of field of the objective used (4x, NA=0.1) was about 50 microns. Since we focused the visceral pleura, the microscopic image referred to a depth of 50 microns within the lung parenchyma. We also performed common histology (hematoxilin-eosin) on sagittal sections of the lung fixed at a transpulmonary pressure corresponding to FRC to estimate the average thickness of the visceral pleura [99].

We imaged the alveolar units of the right lower lobe, where no interlobar fissures crossed the optical field. Alveolar units included both alveoli and alveolar sacs. The change in alveolar size was assessed during two/three recruitment-derecruitment maneuvers: mechanical ventilation was stopped at the end of expiration and lung was recruited from Functional Residue Capacity (FRC) up to an alveolar pressure ($P_{alv}$) of 20 cmH$_2$O (100% of Inspiratory Capacity, IC) by injecting air through a syringe and controlling intratracheal pressure. Each step involved an increase/decrease of 4 cmH$_2$O. During the maneuver, an image was acquired at each pressure step, when quasi-static conditions were reached (about 2 seconds for each pressure level). The displacement of the alveolar field under microscopic view required a manual focus adjustment at each pressure step. At the end of the experiment, the animals were euthanized with an anesthetic overdose.

**Morphological and morphometric analysis of alveolar units.**

For each image, alveolar units (both alveoli and alveolar sacs) were manually segmented along the sharp gradient in optical density between the gas phase and the interstitial tissue in order to derive individual alveolar surface area (Figure 4.3C) and to obtain then their frequency distributions. Given a rectangular Region Of Interest (ROI) on the image, the Alveolar Surface Density (ASD) was estimated as the ratio of alveolar surface area to the area of the ROI; up to four equally sized, minimally overlapping and randomly selected ROIs containing at least 10 alveoli were analyzed for each image to obtain an average value for ASD at each alveolar pressure (Figure 4.3C).

**Alveolar segmentation technique**

To the best of our knowledge, no alveolar segmentation algorithm exists for a single in-vivo microscopic image. The difficulty lies in the fact that alveolar contours are often not completely visible or jagged and are affected by noise and illumination artefacts. Two different algorithms have been proposed to measure the surface area of individual alveoli tracked over time during in-vivo microscopic experiments by the application of gentle subatmospheric suction (~5 cmH$_2$O), allowing to keep the same alveolar region under the microscopic view. These algorithms require an initial segmentation for the first
frame of each alveolus; for successive frame, after image pre-processing based on border enhancement, alveolar border are found either by a shape-matching approach [113] or active contour (snakes) [114].

Figure 4.3 Experimental preparation. A: macrophotography on creating a “pleural window”. (a) intact chest wall, (b) intercostal muscle layer, (c) denuded portion of parietal pleura (“pleural window”). B: part of a pleural window under the microscopic view with a magnification of 60X: alveoli are clearly visible where the endothoracic fascia was stripped off the denuded parietal pleura (c1), as compared to the unstripped portion (c2). C: An example of manually segmented alveolar units (digital zoom of a 60x magnified microscopic image) at $P_{ld} = 4.5 \text{ cmH}_2\text{O}$. Four non-overlapped ROI are shown together with the corresponding percentage values of alveolar surface (Alveolar Surface Density, ASD). D: the same alveolar population of C with manually traced alveolar clusters (black lines) obtained by the contiguity-constrained hierarchical clustering analysis. Alveoli not belonging to any clusters are not encircled. Reprinted from [115].

We applied a method able to overcome issues of non-complete alveolar boundary and noise artefacts, based on the shortest path approach, already applied in [116] for cell segmentation: briefly, as shown in Figure 4.4, for each alveolus a central point and a
boundary point are required; the image is transformed to polar coordinates and the shortest path approach is used to close the boundary.

**Figure 4.4** Alveolar segmentation based on the shortest path proposed by [116]. For each alveolus, an internal (red in the figure) and a boundary (green in the figure) point are needed. The image is converted from cartesian to polar coordinates and the shortest path is applied to find the minimum energy path completing the border. Finally, the image is re-converted to cartesian coordinates.

**Figure 4.5** Iterative voting to obtain internal and boundary points necessary to perform the shortest path approach described in Fig. 4.4. Top row represents the iterative approach providing the gradient-based voting image, as detailed in the text. Briefly, a threshold is applied to the gradient image and triangular kernels are projected starting from supra-threshold pixels. Only summing kernels are left and iteration is performed on left pixels, by reducing angular aperture. Second and third rows show results of the approach on several alveoli. Each iteration step is produced by the sum of kernels and last image shows the obtained internal and boundary points.
For a full automatization, the choice of the central and external points are made by a second algorithm, based on iterative voting (Figure 4.5), an approach used for both astronomic and cellular segmentation [117]. A logical gradient image is obtained computing pixel by pixel the image gradient value and applying a global threshold (second image on the top row of Figure 4.5).

For each white pixel, a triangular kernel function multiplied by the gradient value is projected along the radial direction and each kernel is summed, as shown by the third image on the top row of Figure 4.5, where, for clarity, projections are superimposed on the original image and not on the gradient image.

The approach is iterated on the original image only for those gradient points which, summed to other kernels, provide a sufficiently great value (fourth image on the top row of Figure 4.5). At each iteration, the triangular kernel is progressively reduced in angular aperture (fifth image on the top row of Figure 4.5). After some iterations, a threshold is applied to obtain internal points, while boundary points can be found by looking for maximum value of the original image along the radial direction. Results of the algorithm are satisfactory for rounded alveolar structures, but not for multilobate alveoli (second and third rows of Figure 4.5); therefore, alveolar segmentation was verified for each alveolus before including the alveolar surface area values in results. It should be noted, relative to alveolar segmentation, that the external white border does not represent the real alveolar edge [118], but has been used as an underestimated surrogate throughout the entire analysis for both Chapter 4 and 5.

**Local distending pressure.**

Given the animal position, the imaged lung units of the right lower lobe were placed in the less dependent portion of the pleural cavity, that is at 100% of lung height. For these units, it was possible to obtain an estimate of the local distending pressure ($P_{ld}$) defined as:

$$P_{ld} = P_{alv} - P_{lps}$$

where $P_{lps}$ is the local pleural pressure. $P_{lps}$ was derived from figure 7 of [119], a study that reported direct measurements performed at different lung heights and for different $P_{alv}$ values in the same species.

**Cluster and fractal analysis.**

Cluster analysis was carried out to describe the shape and topological distribution of clusters of alveoli having similar area values and was based on contiguity-constrained hierarchical agglomerative clustering [120]. This analysis allows to group alveoli based on two inclusion principles: the first one is based on similarity in surface area, the second is contiguity. The contiguity constraint is initially computed on single alveoli that are considered adjacent if their centroids are not farthest than two times their average surface diameter and no other alveoli are found on the line joining their centroids. A representative example of this clustering analysis is presented in Figure 4.3D, where
alveolar clusters are manually encircled by black lines. Clusters were geometrically characterized by computing their circularity, defined as \(4\pi \times \frac{\text{area}}{\text{perimeter}^2}\) grouping data at \(P_0\).

**Statistical analysis.**
Kolmogorov-Smirnov normality test was performed on alveolar populations using Matlab®. Spatial statistics (semi-variogram) of areas of single alveoli was performed to express the relationship of the respective variances as a function of increasing distance (“lag”) between individual alveoli, in order to assess whether there is a distance dependence for alveolar area distribution. Logarithms of area values were used to obtain normal distributions, on which semivariogram analysis was carried out, using geoR package (R, version 3.0.1). Semivariogram could be computed only up to a maximum distance corresponding to half the window size (about 500 µm).

### 4.3 Results

**Figure 4.6** Alveolar populations taken at different alveolar pressure during an inspiration maneuver from 0 to 20 cmH₂O.
Figure 4.6 shows several alveolar populations taken at increasing alveolar pressure levels from a single rabbit. It emerges that alveolar expansion occurs in the range $0 - 8 \text{cmH}_2\text{O}$. Results of the analysis will be presented in accord to three scale level: alveolar, cluster of alveoli and overall image/whole lung.

**Alveolar level:** Representative distributions of alveolar areas pooled from all rabbits are presented in Figure 4.7A and Figure 4.7B for $P_{alv} = 0 \text{cmH}_2\text{O}$ and $P_{alv} = 20 \text{cmH}_2\text{O}$, corresponding to local distending pressure $P_{ld}$ of 3 and 16.5 cmH$_2$O, respectively referred, from this point onwards, as $P_0$ and $P_1$. These distributions were found to be right-skewed, that is smaller alveoli are more numerous than the greater ones. The semi-variogram presented in Figure 4.7C shows that the variance of the alveolar areas at $P_0$ is independent of topological distribution, as it oscillates by about ± 3% around the variance of the alveolar population, named “sill”. Similar results were obtained for the same analysis carried out at increased distending pressure, with an average oscillation of ± 7% around the “sill”.

Figure 4.7D shows that the median values of alveolar areas, pooling data from all rabbits, increases with $P_{ld}$, with a remarkably increasing wide variability (expressed by the 25th-75th percentiles, dashed areas). Kolmogorov-Smirnov normality tests failed for distributions at all distending pressures. Also log-normality test, performed by applying normality test on the logarithm of data, failed for each distribution.

The coefficient of variation (CV) of each distribution was computed by dividing the interquartile range (75th percentile value minus 25th percentile value) by the median value and did not show any specific trend to increase with increasing distending pressure: for local distending pressure of 3, 4.5, 8.5, 10.5, 13, and 16.5 cmH$_2$O on the inflation limb, the CV values were 88%, 79%, 87%, 92%, 79%, 80%, respectively.

**Cluster level:** Figure 4.3D allows to appreciate the complex matching of clusters due to their highly variable shape. Figure 4.8 shows that cluster shapes are far from circularity and quite dispersed.

**Whole image level:** The average values of ASD± SD, obtained by pooling values from all animals, is reported in Figure 4.9A and shows an increase up to a $P_{ld}$ of 10 cmH$_2$O, with coefficient of variation at each distending pressure not exceeding 25%. Figure 4.9B shows the overall lung volume versus distending pressure curve and allows to appreciate that the average coefficient of variation of volume is further reduced to about 6%.
Figure 4.7 Alveolar area distributions. A,B: Distributions of alveolar areas at $P_{ld} = 3$ and 16.5 cmH$_2$O ($P_0$ and $P_1$ respectively), obtained by pooling data from all rabbits. C: box plot describing the alveolar surface areas from all rabbits at different levels of alveolar pressure (only the inflation limb is shown). For each pressure, the gray box shows a 25%-75% percentile range; the median value is represented by the horizontal bar. The whiskers above and below the gray boxes encompass 80% of the variability of area values. D: semi-variogram of alveolar areas at $P_0$. Reprinted from [115].

Figure 4.8 Histogram of circularity values for alveolar clusters $P_0$. Reprinted from [115].
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Figure 4.9 ASD and whole lung results. **A**: Alveolar surface density (ASD) as a function of the local distending pressure. Mean values of data pooled from all rabbits are presented with ± SD. **B**: transpulmonary pressure vs lung volume curve (normalized to TLC), obtained as the average of individual rabbit pressure-volume curves. Reprinted from [115].

4.4 Discussion

Previous attempts of imaging of subpleural alveoli to some extent largely interfered with unrestrained alveolar movement [121]. For example, in [99] and [109], the visceral pleura was stuck to a transparent window. By using a tomographic technique as optical coherence tomography [122] or confocal microscopy [123], a three-dimensional (3D) analysis could be performed. In this case, however, less pressure steps could be done in in-vivo experiments due to the time required by the 3D acquisitions.

The experimental approach used in this study allowed to investigate the morphology of the subpleural alveoli by maintaining pleural space mechanics intact. A notable limitation was that it was not possible to follow the morphological change of the same units. On the other side, the method had the advantage to allow unrestrained movement of subpleural units, exposed to physiological distending pressure, by preserving the integrity of the respiratory system. This allowed to relate the morphology of the alveoli, assessed through a two-dimensional analysis, to the expected local distending pressure [119]. This last point is of remarkable importance as it is not available by other techniques used to investigate the morphology of individual alveoli within the lung parenchyma.

Another potential limitation is represented by the fact that only subpleural units were studied and the discussion concerning their mechanical behavior does not allow us to extrapolate to the rest of the parenchyma. In fact, the areas of the subpleural alveoli are controlled by the lines of attachment of alveolar walls to the pleura and the displacements of these attachments relate to the displacement of the pleural membrane which has been shown to expand isotropically as lung volume increases [124]. However,
recalling data from [20], no differences in surface area expansion have been described between subpleural and core alveoli from fixed rabbit lungs. Finally, our imaging system did not allow a 3D reconstruction.

Given the depth of field of our objective (50 microns), we essentially imaged the terminal airways that may include both alveoli as well as alveolar sacs, as confirmed by sagittal histological sections from our lab; as specified in the methods, we only considered surface area values of the alveoli. One may wonder whether the observed heterogeneity could in part be due to the fact that the focal plane cuts the alveoli at different positions with respect to their centroids. This would lead to an error in the measured areas, representing an underestimate relative to the area corresponding to the plane of the alveolar centroid. From the histological sections, we also estimated an average thickness of the visceral pleura of 10±2 microns, so that all alveolar walls are essentially at equal distance from the pleural surface. Therefore, the underestimate would only occur for larger alveoli when the centroid is much below the focal field. This bias, however, is presumably constant at all pressures, so that it would not affect relative changes in alveolar areas observed in increasing distending pressure.

Distribution of alveolar areas were found to be right-skewed, meaning that small alveoli are more numerous than larger ones; this fact could be explained by hypothesizing that the larger alveolar units included in the analysis probably represent aggregates of adjacent alveoli (alveolar sacs) appearing on the subpleural surface. By considering the histograms of Figure 4.7A, we will attempt an interpretation of the change in distribution of the alveolar caliper with increasing $P_{ld}$ to derive indications on the mechanical properties of the alveoli. In particular, we propose to interpret our data in terms of alveolar distensibility. The latter property was estimated by the ratio of the increase in alveolar surface area for an increase in alveolar distending pressure from $P_0$ to $P_1$, namely $C_{abs} = (A_1 - A_0)/(P_1 - P_0)$, which represents an average index of alveolar absolute compliance in the range of pressure considered. We also defined specific alveolar compliance as $C_{sp} = C_{abs}/A_0$.

As we cannot generally follow the same alveoli on increasing $P_{ld}$, we attempted to derive both absolute and specific alveolar compliance from the frequency distribution of alveolar areas at $P_0$ and $P_1$. We implicitly assume that, within a frequency distribution at a given $P_{ld}$, alveoli of different size are actually all exposed to $P_{ld}$, for mechanical equilibrium. We defined subpopulations from the frequency distribution at $P_0$ and $P_1$ by dividing the overall populations in 50-quantiles and we assumed that each $i$-th subpopulation at $P_0$ fell in the corresponding $i$-th subpopulation at $P_1$. The absolute compliance of the $i$-th subpopulation, $C_{absi}$, from $P_0$ to $P_1$ was estimated from the increase in median value of the subpopulation itself $(\bar{A}_{1i} - \bar{A}_{0i})$, divided by the increase in $P_{ld}$:

$$C_{absi} = \frac{\bar{A}_{1i} - \bar{A}_{0i}}{P_1 - P_0}$$
The specific compliance index was given by
\[ C_{sp_i} = \frac{C_{abs_i}}{\bar{A}_0} \]

Figure 4.10A shows that the computed estimate of \( C_{abs} \) (continuous line), increases seven-fold for a range of increase of \( A_0 \) including 90% of alveolar population. Correspondingly, one can appreciate that specific compliance \( C_{sp} \) (Figure 4.10B continuous line) remains essentially steady (0.12 ÷ 0.15 cmH\(_2\)O\(^{-1}\)) for the same range of alveolar population. To simplify the model, we assumed a constant value for specific compliance, reported by the dashed lines in Figure 4.10B, as given by
\[ \bar{C}_{sp} = \frac{(\bar{A}_1 - \bar{A}_0)}{(P_1 - P_0)/\bar{A}_0} \]

(where \( \bar{A}_1 \) and \( \bar{A}_0 \) are the median value of distributions at \( P_0 \) to \( P_1 \)). Obviously a constant value for \( C_{sp} \) implies a linear increase in \( C_{abs} \) (Figure 4.10A, dashed line). To validate this assumption, we show in Figure 4.10C that a similar satisfactory modelling of the experimental distribution (white points) at \( P_1 \) was found considering either the variable or fixed specific compliance (dashed black and gray line, respectively). One shall comment that the derived absolute compliance values obviously reflect the shape of the experimental area distribution curves and the modelling assumption of the correspondence of the \( i \)-th subpopulations at the various \( P_{ld} \).

**Considerations relative to the difference in the mechanical behavior of lung units.**

Results of this study reveal that the considerable heterogeneity in alveolar size (Figure 4.10C) does not show a topological dependence, as demonstrated by the semi-vario gram of Figure 4.7D. It indicates that, at \( P_0 \), variance increases asymptotically within the first 100 \( \mu \)m, a distance covering about two average alveolar diameters; this fact may be interpreted in terms of mechanical interdependence among adjacent alveoli, sharing the same septal wall and probably exchanging surfactant. Mechanical interdependence could be studied by analyzing the same adjacent alveolar unit during recruitment/de-recruitment maneuvers and/or in conditions of altered mechanical conditions (surfactant deprivation, destruction of the elastic structural component). At higher distances, topological dependency of similarity in alveolar area vanishes, indicating that heterogeneity results to be homogenously distributed. This is confirmed by the fact that similarity-based alveolar clusters, possibly describing alveoli subtended by the same airway) also appear to be variously interweaved (Figure 4.3D) having a highly variable shape (Figure 4.8).

Obviously, we are far from extending our considerations to the whole lung functions where inhomogeneous behavior is known to be present concerning various aspects such as gas mixing, regional volume change, due to several interacting factors, such as gravity, airway resistance, etc. Interestingly, scaling up from single alveoli to randomly selected alveolar regions, a relatively homogenous mechanical behavior with minimal hysteresis of overall alveolar expansion is observed, as suggested by the low coefficient of variation of ASD (Figure 4.9A). This occurs despite the fact that differences in
alveolar morphology imply differences in absolute compliance (Figure 4.10). Scaling up to the level of the whole lung, a relative homogenous behavior is also observed given the low coefficient of variation of lung volumes at various transpulmonary pressures. Note that for the whole lung expansion, various structures with different mechanical properties become involved (alveolar ducts, bronchioles, bronchi). In fact, on comparing Figure 4.9A and B, one can deduce that above 10 cmH₂O a contribution to increase in lung volume must come from lung structures other than the alveoli under analysis and this also implies an increase in hysteresis from ASD to whole organ organ, confirming previous findings obtained on isolated lung lobes using a synchronized stroboscopic photography [124].

In conclusion, to answer the questions addressed in the introduction, our data suggest that the mechanical behavior found by scaling up to lung units and to the whole organ

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**Figure 4.10** Estimation of alveolar compliance. **A**: Absolute compliance estimated by comparing subpopulations drawn from alveolar area distributions at $P_0$ and $P_1$ (continuous line). **B**: Specific compliance derived from the absolute compliance, as detailed in the text (continuous line). Dashed line in Panel A and B correspond to the assumption of a constant specific compliance. **C**: Modelling of the experimental alveolar area distribution at $P_1$ (white dotted points), assuming either a variable (black dashed line) or fixed (gray dashed line) specific compliance. Reprinted from [115].
results from a non-topological dependent morphological and mechanical heterogeneity at micro-scale level (alveoli and cluster). The relative constancy of the estimated alveolar specific compliance could be an important factor to account for morpho-functional homogeneity on increasing scale level. Indeed, we might consider that, in an interdependent environment, a homogenous distribution of specific compliance of elastic components might reduce interregional differences in parenchymal forces during lung volume changes. Of course, this working hypothesis requires an experimental validation by following the expansion of the same alveoli on increasing distending pressure. Regarding the fact that no hysteresis was observed in alveolar areas, we can make the following comment: on one side, this may be due to the fact that we could actually not follow the same alveoli; on the other, we may suppose that hysteresis arises from other structures such as alveolar ducts. The hysteresis observed in lung volume change, despite a small CV of lung volume (Figure 4.9B), may be ascribed to a multiscale effect of hysteretic factors on lung units having different mechanical properties. This can be the object of a further study requiring the use of imaging techniques allowing to explore internal parenchymal structures.

4.5 Conclusions

1) The present study investigates pulmonary alveolar mechanics in closed-chest rabbit lung by using an animal preparation allowing unrestrained movement of alveoli.  
2) In the condition of unrestrained movement, subpleural alveoli show an isotropic increase of surface area for an alveolar pressure ranging from 0 up to 8 cmH\textsubscript{2}O. Further increase of distending pressure does not result in alveolar expansion, thus involving the recruitment of other micro-structural components (alveolar ducts, internal alveoli). 
3) Subpleural alveolar areas does not show a topological dependence on adjacency, indicating that heterogeneity of alveolar areas is homogenously distributed. 
4) Alveolar expansion is proportional to baseline alveolar areas; this constant specific alveolar compliance may be the basis of the greater homogeneity of mechanical behaviour found by scaling up from alveoli to the whole lung. 
5) In vivo microscopy is an useful tool to investigate lung peripheral micro-structures.
Appendix. Experimentally measured absolute and specific alveolar compliance

A careful observation of images throughout recruitment maneuvers allowed to find the same alveoli imaged at different pressure levels. In particular, in [112], we analyzed alveolar morphology of 60 alveoli at both 4 and 8 cmH$_2$O of alveolar pressure. As shown by Figure 4.11, absolute alveolar compliance positively significantly correlates (P=0.001) with the value of baseline alveolar size (panel A) but absolute compliance is not distributed normally, due to the higher abundance of smaller alveoli and thus of smaller absolute compliance values. As a consequence, the specific compliance does not show any linear trend with respect to baseline alveolar size (Figure 4.12).

![Figure 4.11](image.png)

**Figure 4.11** Absolute compliance for tracked alveoli. A: Absolute alveolar compliance computed in the range 4-8 cmH$_2$O plotted versus alveolar size at 4 cmH$_2$O. B: Distribution of absolute compliance for all alveoli considered. Reprinted with permission from [112].

Alveolar aspect ratio was also analyzed; this morphological index is a shape factor defined as the ratio major diameter/minor diameter, and can be considered as an index of alveolar deformability. Figure 4.13 shows that at lower distending pressure, the aspect ratio and thus alveolar deformability is proportional to alveolar size, a result in line with the higher absolute compliance of large alveoli (Panel A). Increasing distending pressure, the aspect ratio is more homogenous, with the smaller alveoli becoming more distensible. This finding can be explained by considering the force balance involved in alveolar stability, briefly described in Paragraph 1.4; alveolar pressure is counteracted by an elastic tissue component $P_{el}$ proportional to distension and a surface force $P_{γ}$ depending on alveolar radius $r$ and on surface tension, the latter being inversely proportional to the concentration of surfactant on the alveolar liquid layer lining alveolar surface: $P_{γ} = 2γ/r$ (Laplace's law).
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Therefore, the decrease in deformability of large alveoli could be explained by a thinning of the surfactant layer due to mechanical expansion, producing an increase in surface tension and thus resulting in less distensibility; on the other side, surfactant may be redistributed from large to small alveoli due to Marangoni flow [111], resulting in more distensible alveolar structures.

Figure 4.12 Specific compliance for tracked alveoli. A: Specific alveolar compliance computed in the range 4-8 cmH\textsubscript{2}O plotted versus alveolar size at 4 cmH\textsubscript{2}O. B: Distribution of specific compliance for all alveoli considered. Reprinted with permission from [112].

Figure 4.13 Alveolar aspect ratio. A: Box plot of Aspect Ratios at 4 cmH\textsubscript{2}O for three alveolar subpopulations, stratified by baseline alveolar size A\textsubscript{4}. B: Box plot of Aspect Ratios at 8 cmH\textsubscript{2}O for three alveolar subpopulations, stratified by baseline alveolar size A\textsubscript{4}. Reprinted with permission from [112].
5. Regulation of alveolar micro-vascular perfusion and interstitial fluid balance

The content of this chapter will be submitted as part of an original research article to the *Journal of Lung Cellular and Molecular Physiology.*
5.1 Introduction

While in the systemic circulation hypoxia causes vasodilation due to the increased metabolic demand [125], in the pulmonary circulation the adaptive response consists in a vaso-constriction of both pre-acinar, intra-acinary artery [126] and extra-acinar veins [127]. First evidence of vasoconstriction in smaller intra-acinar arterioles were found in dog lung [128], by using a videomicroscope system on isolated and perfused canine lobes. Significant artero- and veno-constriction was found at different pressure levels, therefore implying a contribution of muscular media surrounding the microcirculation. No evidence of blood redistribution was observed, even if hypoxia-induced change in flow was analyzed only in the peripheral-to-distal axis and not on the cranio-caudal direction.

In a different animal model and preparation (intravital microcirculation study on rats) [129], it was found that the principal site of intra-acinar vasoconstriction is represented by the medium-sized arterioles (30-50 µm), while smaller arterioles and intra-acinar venules do not change their diameter in response to hypoxia. This different behaviour of the post-capillary compartment may be explained by the lack of muscle cells in venules of <50 µm in murine circulation, even if the existence of muscular venular sphincters have been identified at the junction between capillaries and pulmonary veins [130]. Relatively to arterioles, smaller vessels may contain contractile pericytes, thus a decreased adaptive response compared to larger arterioles may reside in the muscular tone and vasoactive response of this pre-capillary compartment.

If venular vasoconstriction is still under debate, another well-established hemodynamic effect of hypoxia administration is capillary recruitment, which occurs notwithstanding the vasoconstriction effect: in [51], the capillary recruitment index (CPI), defined as the total capillary length within a field area of 10000 µm², was found to increase 5 times after 15 minutes of alveolar hypoxia, supported by a doubling of pulmonary artery pressure and a constant left atrial pressure. This result implies that pulmonary arterial pressure is the main cause of capillary recruitment, even if at odds with the hypoxic-induced artero-constriction. A possible explanation may reside in the blood redistribution from dependent lung regions due to the generalized arterial constriction: increased blood flow in correspondence of vasoconstriction rises inlet capillary pressure. Anyway, capillary recruitment and blood redistribution from the bottom lung to the upper regions represent an adaptive response aiming at increasing the pulmonary diffusing capacity, as experimentally proven [131], at least in dog model.

Relative to human, a magnetic resonance technique named arterial spin labelling has been applied to quantify blood flow and obtain regional maps of lung perfusion. By considering only the relative dispersion (standard deviation divided by mean mean) measured on the same slice during time, no evidence of increased heterogeneity was
found after hypoxic stimulus [132]. However, by computing the spatial-temporal heterogeneity obtained by image registration, identified as fluctuation dispersion, a significant increase of this parameter was found after hypoxic administration with respect to hyperoxia and baseline treatment [133], thus demonstrating that blood redistribution occurs also in humans.

All the previously cited studies focus on the short term effect of hypoxia administration, but it is of interest to evaluate the long term effect implying also the mechanical effect of edema development. In fact, beside active response, increased interstitial pressure may exert a passive mechanical compression on microvessels, even if in the early stage of hypoxia interstitial pressure does not reach atmospheric pressure [85]. An histological study found that interstitial edema does not influence the caliber of either small (<400 µm) arteries or airway, but also that hydrostatic edema causes an increases in pulmonary resistance, which could be explained by an increase in alveolar and pericapillary edema [134]. The hypothesis of a mechanical effect of edema perivascular cuffs on vascular resistance has been put forward also in an interesting study on human lung aimed finding an increase in perfusion heterogeneity after intense exercise, not coupled by an equal increase of specific ventilation heterogeneity [135].

The aim of the present study is to analyse both the short term and long term effect of an edemagenic stimulus, namely hypoxia administration, on micro-vascular dynamics and on capillary recruitment; also, the effect on alveolar mechanics and on capillary corner will be evaluated. The model described in Chapter 3 will be applied to the images in order to derive indications about arterial and venular vasoconstriction. The hypothesis is that interstitial tissue mechanics and microvasculature are mechanically inter-dependent systems able to provide functional adaptive responses to edemagenic conditions.

5.2 Methods

A general consensus for the experimental procedures used in our research activity was obtained from Milano-Bicocca University Ethical Committee. Four adult male New Zealand White rabbits (weight range 1-1.5 kg) were anesthetized, tracheotomized, paralyzed and mechanically ventilated following the same procedure described in Chapter 4 and in [112]. Subpleural alveoli were visualized through a "pleural window" obtained following the same surgical procedure described in Chapter 4; the pleural window was prepared in the seventh intercostal space which allowed to visualize at end expiration the highly vascularised lobar margin. Images were acquired using a Nikon SMZ stereo microscope, equipped with a CMOS camera (OPTIKAM-B5). A magnification of 15X was used, as a compromise allowing a resolution of 7.22 µm; this magnification was chosen as a compromise between sufficient resolution and a field of
view large enough to track the same subpleural region throughout the experiment. A LED ring-light illuminator was anchored to microscopic optics to provide a uniform lighting of the alveolar field.

**Experimental setup**

**Hypoxia protocol:** After baseline imaging of subpleural micro-vascular and alveolar morphology, rabbits received a hypoxic mixture (12% oxygen and nitrogen) throughout the whole experiment. Images were then acquired every 10 minutes in the first hour and every 20 minutes for the following 120 minutes, at both end expiration and end inspiration. During the experiment, the same subpleural regions during end-expiration and end-inspiration could be imaged, so that the same alveolar units and the same pre-capillary vessels could be tracked. Tidal volume of mechanical ventilation was set as to provide the same peak inspiration alveolar pressure throughout the whole experiment (12 cmH$_2$O). At the end of the experiment, animals were euthanized by anaesthetic overdose, thorax was opened and some lung samples were cut and weighted. They were desiccated in oven at 60° for 2 days and then the dry weight was determined to obtain the wet-to-dry ratio.

![Figure 5.1 Positioning stage for respiratory tracking. The vertical stage is motorized to allow compensating the respiratory movement, based on the ventilator inspiratory flow.](image)

*Positioning stage:* A positioning stage (Figure 5.1) was developed to track the subpleural region during the respiratory cycle. The 3-axis stage is shown in Figure 5.1 and is based on three micrometers allowing a 1-cm moving range; vertical displacement was automated by motorizing the corresponding micrometer. Initially, end expiration and end inspiration focal depth were calibrated; during the experiment mechanical ventilatory flow was acquired to trigger the change of position, the stepper motor maximum velocity was sufficient to follow subpleural lung surface within the same breath. Unfortunately, lung sliding under the pleural window did not allow to track the same region between end-expiration and end-inspiration, since no suction device was used to leave unrestrained movement to the subpleural region.
Image analysis

Alveolar segmentation: Alveolar units were manually segmented, by following their borders identified by the gray level between air and tissue phase. The distance of alveolar border from the closest distribution vessel was also measured. 420 alveoli were included in the analysis and alveolar segmentation was performed in 3 time points (Baseline, 40 and 120 minutes).

Segmentation of distribution vessels: Distribution vessels are distinguished from corner vessels based on two criteria, based on visual inspection of a large dataset of images and on the definition of corner vessels provided by [13]: 1) distribution vessels should have a diameter greater than 30 µm and 2) a length between two successive bifurcations greater than 100 µm. Segments of vascular trees are analyzed separately considering each segment individually. Vascular diameter and interstitial thickness are evaluated using the semiautomatic procedure described by [136], developed in Matlab. Briefly, standard deviation (sigma) image and standard error (tau) image on a 7X7 pixel large moving window are obtained from the original image (Figure 5.2).

Figure 5.2 Image preprocessing for vascular segmentation. Standard deviation (B) and standard error (C), based on a 7x7 pixel large moving window are computed on the original image (A).

For each point along the vessel longitudinal length, the vessel border is identified as the first peak of sigma along the normal direction; interstitial compartment is considered to be delimited by the following peak of tau profile along the normal direction (left panel of Figure 5.3). Each vessel segment is described by its average vessel diameter and average perivascular interstitial thickness. Due to image resolution and gaussian noise affecting interstitial segmentation, only results about vascular dynamics on 28 distribution microvessels will be presented. Distribution vessels were visible only at end expiration, while end-inspiration presented alveolar texture with clear corners, but no evidence of distribution vessels. A control group of 12 distribution vessels was imaged, with a sampling rate of 20 minutes, for an hour, during which no treatment was applied to the animal.

Corner vessels segmentation: Vessels surrounding alveolar profiles, not matching criteria for distribution vessels, are manually analyzed in terms of average caliber. Corner compartment represents the vascular network directly feeding the septal network which could not be imaged with our optical system. This definition of corner vessels has
already been proposed by [13]. Image analysis was performed using ImageJ. Analysis results were used also for modelling purposes (see next paragraph).

**Figure 5.3** Vascular segmentation method. Left panel shows the three normalized profiles of the original image (blue line), of the standard deviation image (red line) and standard error image (black line), measured along the normal direction of a representative distribution vessel (shown in right panel, where the normal direction is identified by the green line). First left and right local maxima of the standard deviation profile, with respect to the vessel centre, provide the vessel borders, while the first left and right local maxima of the standard error image are interpreted as the outer borders of the interstitial compartment. Segmentation results is shown in right panel, where blue line identifies vessel borders and yellow line the limit of the interstitial compartment.

**Model calibration on experimental data**

**Building 3D model**

In rat, as well as in the peribronchial space, subpleural surface has been demonstrated to have smaller capillary density within the lung parenchyma [14] [11]: only 73% of subpleural and peribronchial alveolar surface is covered by capillaries against a 86% density of internal septal structures. Notwithstanding this lower density, subpleural alveoli images reveal a clear distinction between corner and septal networks, as it has been noted by [13]. In our case, image resolution did not allow to visualize internal septal capillary laying on the alveolar surface, which mean caliber size (5.5 µm, according to [14]), is less than pixel size (7.22 µm).

Based on the images, the alveolar-capillary model described in Chapter 3 was adapted to visible networks in the following way: the same alveolar region is identified during the same experiments, involving about eight to ten alveoli. For each network, the following dataset was obtained: 1) coordinated of nodes, 2) diameters of corner vessels measured throughout the experiment: $R_i(t)$, representing the average corner radius measured at time $t$. 

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**Chapter 5**
Figure 5.4 shows the workflow to build a three-dimensional (3D) model based on the previously collected data. At first, a two dimensional (2D) network was built: corner segments surround alveolar septal facets containing septal capillaries, whose densities are based on the data of [11]; 2D network construction follows the same approach illustrated in Chapter 3. A second 2D network was connected by vertical projections so as to define alveolar cuboidal structures.

Several assumptions were made in order to build the final 3D model:

1) Since 3D OCT (optical coherence tomography) highlighted a cuboidal shape for the first layer of subpleural alveoli [137], we adopted this simplified alveolar model.

2) Arteriolar access points represent the terminal part of visible subpleural arterioles.

3) Venular exit points are located on the floor of the first alveolar layer and were assigned to nodes presenting the aspect of a venular collecting lacuna.

4) To reduce computational effort, the sub-net was assumed not to interact with adjacent networks; with this assumption, we hypothesize that boundary corner segments do not exchange fluid with non-represented corner segments external to the network.

5) Non-visible corner vessels were assigned a diameter value equal to the resolution limit of the image (7 µm), with a random variability of ±5%. No measures are available for corner vessels in literature, but by assuming an average septal radius of 5.5 µm [14], and a corner-to-septal radius ratio of 1.32 [69], 7 µm appears to be a reasonable value.

6) A value 100 µm of alveolar height was chosen (equal to an average alveolar radius for rabbits).

7) Septal capillary segments undergo the same passive relationship expressed by Eq. 3.2, therefore average septal radius is about 3 µm.

Once defined the 3D model topology, it was possible to apply the same procedure described in Chapter 2 and Chapter 3 to compute capillary hemodynamics. The input hemodynamic parameters are three: inlet arteriolar pressure \( P_{art} \), outlet venular pressure \( P_{ven} \) and interstitial liquid pressure \( P_{liq} \).

The rationale for this analysis was to derive semi-quantitative information about the changes in input arteriolar pressure and the output venular pressure due to hypoxia administration, which could provide indications about the occurring functional adaptive response (arterial vaso-constriction, increase in arteriolar pressure due to blood redistribution, venular vasoconstriction). Not all time points were analyzed, but only those representative of the functional phase identified by the analysis of vasoactive response of distribution vessels.
Baseline network was taken as reference for successive time points. In particular, for each time point considered in the analysis, the interstitial liquid pressure was derived from values experimentally measured by micropuncture technique \cite{85}. Relative to $P_{\text{art}}$ and $P_{\text{ven}}$, the following approach was used: for baseline a value of $P_{\text{art}} = 16 \text{ cmH}_2\text{O}$ and $P_{\text{ven}} = 6 \text{ cmH}_2\text{O}$ as experimentally measured \cite{138}. From the baseline solution, it was possible to obtain the pressure values for each $i$-th corner vessels $R_{i\text{baseline}}$.

All the networks built on subpleural regions were taken from images at end expiration. As detailed in Table 5.1, successive time points were analyzed with different combinations of $P_{\text{art}}$ and $P_{\text{ven}}$, with the latter ranging from 3 and 9 cmH$_2$O (by step of 3 cmH$_2$O) and the former ranging from 12 and 21 cmH$_2$O (also by step of 3 cmH$_2$O). Each possible solution $k$ for each time point $t$ provide an estimation of transmural pressure for each $i$-th corner segments $\{\{P_{i,j}\}_t\}_k$.

**Figure 5.4** Workflow to obtain the 3D pulmonary capillary network by an in-vivo microscopic image. Both visible alveolar corner capillaries (red lines), surrounding alveolar structures, and non-visible septal capillaries (blue lines) are shown. The septal capillary network, not visible in the image due to resolution, is built based on morphometric data, by following the approach of Chapter 3; the model is extended in three dimensions by following some assumptions (detailed in the text). Arteriolar access points (red circles) are taken over visible arterioles, while venular exit points (blue circles) are assigned to the alveolar bottom floor in correspondence of blood lacunae.
Therefore, for each solution it was possible to compute the correlation between the increase/decrease in average corner radius (measured from images) and the increase/decrease in corner pressure (computed from the model) with respect to baseline:

\[ R_{iBaseline} - R_i \text{ vs } P_{iBaseline} - P_i \]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline*</th>
<th>Successive time points</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_{art}[cmH_2O] )</td>
<td>16</td>
<td>12 (-÷20) discretized range</td>
</tr>
<tr>
<td>( P_{ven}[cmH_2O] )</td>
<td>6</td>
<td>3 (-÷9) discretized range</td>
</tr>
<tr>
<td>( P_{inter}[cmH_2O] )</td>
<td>-10</td>
<td>-10-3 (taken from [85])*</td>
</tr>
</tbody>
</table>

Table 5.1 Input parameter choice for image-based ACU modelling.

The idea is that, since alveolar corners are not surrounded by annular muscular structures as small arterioles, their dimensional changes over time should be related to changes in transmural pressure. Therefore, the optimal solution is the one that maximizes the mechanical correlation between vascular pressure and caliber size.

**Statistical analysis**

2-ways RM ANOVA with independent variables being time and baseline vessels was applied on the absolute values of arteriolar calibers to estimate the dependence of vasoactive response of distribution microvessels on time of administration and on vessel size. The correlation between interstitial pressure and corner radius was evaluated by a 2-way Repeated Measurement (RM) Analysis of Variance (ANOVA), with independent variables being time and end inspiration/end expiration condition. Three 2-ways Repeated Measures (RM) analysis of variance was also performed to assess 1) the dependence of alveolar size on both time of treatment and the end-expiration versus end-inspiration condition, 2) the dependence of alveolar size on both time of treatment and alveolar distance from the nearest distribution vessel (only for end-expiration alveoli, since no distribution vessels were found in the end-inspiration alveoli) and 2) the dependence of alveolar size on both time of treatment and baseline alveolar size (two categories of alveoli were considered: small and large alveoli, having a baseline alveolar size respectively less and more than 15000 µm²).

5.3 Results

**Wet-to-dry ratio**

Wet-to-dry ratio was 4.91±0.14, while control value for rabbit lung was 4.3±0.72 (measured on 9 rabbits in a previous experiment from our lab [139]). T-test performed on the two populations provides no statistically significant difference (P=0.092), at least by assuming a significance level of 0.05; from the same experiment, the wet-to-dry ratio for saline intravenous injection was 5.23±0.59 [139].
Distribution vessels
Box plot representation of vessel caliber distributions normalized to baseline are represented in Figure 5.5. An overall statistical difference is found among different time points (p<0.001) through 1-way RM ANOVA; the time pattern reveals the existence of two different functional phases. PHASE I is characterized by a strong vasoconstriction: first 40 minutes after hypoxia administration reveal a significant decrease of vessel caliber, with 10 over 28 vessels becoming totally non-perfused in the first time point (10 minutes), while the maximal peak of vasoconstriction is reached around 30 minutes after hypoxia administration. One hour after the beginning of hypoxia administration, a PHASE II can be identified: perfusion is re-established at levels significantly different from that of the first hour. Vessel calibers increase significantly with respect to PHASE I and also to Baseline even if not significant. Relative to the first hour of control, no statistical difference was found by 1-way ANOVA, as shown by the left panel of Figure 5.5.

![Box plots of distribution vessel diameter normalized to baseline values.](image)

**Figure 5.5.** Box plots of distribution vessel diameter normalized to baseline values. The solid red line links the mean values of each distribution. * P<0.001 vs Baseline § P<0.001 vs 10, 20, 30, 40 minutes. † P<0.001 vs 10, 20, 30, 40, 50 minutes.

Corner vessels
Figure 5.6 shows the absolute variation of 164 corner calibers in µm compared to baseline, in PHASE I and PHASE II. 2-ways RM ANOVA, performed with respect to temporal phase and to the endexpiration versus endinspiration condition, highlights a significant decrease of corner calibers from baseline to PHASE I and significant increase from both baseline and PHASE I to PHASE II (all these differences having a Pvalue<0.05). A statistically significant difference was found from the larger end-

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expiration and the narrower end-inspiration corner vessels (P<0.05), but no interaction between the phase of treatment and the end-inspiration/end-expiration condition was found.

Figure 5.6 Corner caliber variations with respect to baseline in PHASE I and PHASE II. 2-way ANOVA analysis performed on corner calibers with respect to time and end-inspiration/expiration condition revealed a significant decrease (*P<0.05 versus baseline) for all conditions in PHASE I and a significant increase in PHASE II (§P<0.05 versus baseline and PHASE I).

**Alveolar mechanics**

420 alveoli, taken at both end-inspiration and end-expiration, were analyzed. 2-way ANOVA performed on absolute values of alveolar areas found a significant difference among time points (P<0.001), but no specific difference between single time points was revealed. Relative to the influence of the end-inspiration/end-expiration condition, Figure 5.7A shows a significant increase of alveolar size was found between baseline and 40 minutes (P<0.05), and baseline and 120 minutes (P<0.05) for end-expiration. At end-inspiration, alveolar size significantly increased only between 120 minutes and 40 minutes. A significant increase of alveolar size was found for all phases between end-expiration and end-inspiration; furthermore, by evaluating alveolar size at baseline, the increase was more markedly pronounced for bigger alveoli (P<0.05) (Figure 5.7B). Finally, no statistical influence of alveolar distance from the nearest distribution vessel was found on alveolar size change after hypoxia administration (not represented).
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Figure 5.7 Alveolar area variations with respect to baseline in PHASE I (40 minutes) and PHASE II (120 minutes). A: 2-way repeated-measures ANOVA analysis performed on alveolar areas with respect to time and end-inspiration/expiration condition revealed a significant increase (*P<0.05 versus baseline) for end-expiration in both phases, and a significant increase from PHASE I to PHASE II (§P<0.05 for end-inspiration). B: 2-way repeated-measures ANOVA analysis performed on alveolar areas with respect to time and small-large baseline alveolar area revealed a significant increase (*P<0.05 versus baseline) for large alveoli (this difference is not specific for any of the two phases, since no interaction was found between baseline alveolar size and time of treatment).

Model calibration
Figure 5.8 shows a representative example of the same ACU network presented in Figure 5.4 (Net 5 of Table 5.2) at the three different phases, in which the same color code scale was used for blood capillary flow. PHASE I and PHASE II ACUs represents the optimal solution obtained by the approach detailed in the Methods section. At baseline, almost the alveolar network of the entire ACU are recruited, with a septal recruitment amounting to 55%. PHASE I is characterized by a strong derecruitment of both the corner and septal network, with only 8% of capillary network available for gas exchange and fluid filtration. Finally, a strong reperfusion for this ACU can be observed in PHASE II, recovering almost an half of the ACU network (50% of capillary recruitment), with increased blood flow and increased septal to corner ratio, despite the high interstitial pressure.
Figure 5.8 A representative network (Net 5) presented at the three different phases; for PHASE I and PHASE II the optimal solution is presented. PHASE I presents a clear de-recruitment of the whole network and a subsequent decrease of blood flow. PHASE II shows an increase of blood flow and coupled by a moderate decrease of septal recruitment. Blood flow scale is the same for all the three networks.
<table>
<thead>
<tr>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>N5</th>
<th>N6</th>
<th>N7</th>
<th>N8</th>
<th>Median</th>
<th>IQR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHASE I</td>
<td>21.0</td>
<td>12.0</td>
<td>15.0</td>
<td>12.0</td>
<td>12.0</td>
<td>15.0</td>
<td>15.0</td>
<td>21.0</td>
<td>12.0</td>
</tr>
<tr>
<td>PHASE II</td>
<td>21.0</td>
<td>12.0</td>
<td>12.0</td>
<td>21.0</td>
<td>21.0</td>
<td>21.0</td>
<td>21.0</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>PHASE I</td>
<td>3.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
</tr>
<tr>
<td>PHASE II</td>
<td>3.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Table 5.2 Optimal solutions and the hemodynamic changes compared to baseline for PHASE I and PHASE II for all the considered networks. Median and interquartile range (75th-25th percentile) values for each hemodynamic parameter are shown.
Table 5.2 shows the hemodynamic parameters for the optimal solution of each network and the median and interquartile range (75th-25th percentile) value for each obtained hemodynamic variable, since data are not normal. Arteriolar vasoconstriction in PHASE I is confirmed by the decrease of the arteriolar pressure, while venular vasoconstriction was found for 6 of the 8 network. PHASE II presents more heterogeneous results for arteriolar and venular vasoconstriction. Septal de-recruitment is observed for both PHASE I and PHASE II, even if with a great extent for PHASE I. Blood flow is reduced in PHASE I, while, notwithstanding septal de-recruitment, is preserved in PHASE II, due to massive corner recruitment and increased arteriolar-venular pressure gradient. As a consequence, even if within a great variability, ACU resistance increases in PHASE I and decreases in PHASE II.

5.4 Discussion

This study provides an integrative view of the mechanical changes at the level of the pulmonary microcirculation, alveolar structure and interstitial compartment occurring in response to an edemagenic perturbative stimulus, namely hypoxia administration for both short-term and mid-term period after the beginning of the treatment. Results of distribution vessels allow to identify three different functional phases (Figure 5.5): a pre-hypoxia control condition, an early adaptive response characterized by a marked arterial vasoconstriction of both distribution and corner vessels, and, as suggested by mechanical modelling, a possible veno-constriction (PHASE I) and a final functional phase with a substantial reperfusion of distribution and corner vessels, accompanied by an increase in interstitial pressure ([85]).

Corner vessels follow the same mechanical behaviour of distribution vessels (Figure 5.6), with the difference that corner vascular perfusion depend only on passive mechanical factors, due to the reduction of the capillary caliber. Significant reduction of corner calibers in PHASE I was found both at end expiration and end inspiration, thus suggesting that, even if no evidence of arterioles feeding the capillary network on the subpleural surface was found at end-inspiration, the adaptive response of arteriolar vasoconstriction should occur independently from perivascular alveolar pressure. Comparatively for the whole organ scale, arterial vasoconstriction was found at both low and high perfusion pressure [128]. Relatively to indications provided by the wet-to-dry ratios, the obtained mean value of 4.9 lies in the range of the mild phase of interstitial edema, characterized by no evidence of fluid flooding into alveoli [139].

Relative to mechanical interaction between alveoli and vascular perfusion, the present study presents the first analysis evaluating in-vivo effect of hypoxia administration on alveolar mechanics (Figure 5.7); alveolar mechanics in edemagenic condition have been already studied in isolated and perfused rat lung [28], showing a mechanical
interdependence between liquid-filled and adjacent air-filled alveoli. However, the mechanical structural changes occurring during mild edemagenic conditions, such as hypoxia, concern mainly the interstitial matrix surrounding alveolar septa and the force-bearing components, and not the surface tension at the air-liquid alveolar surface. An interesting correlation was found, providing the information that the modification of parenchymal microstructure due to fluid accumulation and to arteriolar, corner and possibly venular vasoconstriction may affect the alveolar mechanical equilibrium, mainly related to structural factors. The observed variations in alveolar mechanics should be ascribed to the hypoxic treatment; in fact, in an in-vivo microscopy study from our lab, no significant variation of alveolar caliber was observed [140].

As well as results of Chapter 4 for subpleural alveolar mechanics cannot be extended to the internal microstructure without some considerations, the extension of the behaviour of subpleural distribution microvessels to the internal ones is not straightforward, due to the lower density of surface capillary networks [11] and of different mechanical conditions. However, several studies reported a correspondence between peripheral and internal vascular responses, relatively both to recruitment patterns [141] and blood redistribution phenomenon [142] in normal conditions. Yet, more investigations are required; for example, the latter study [142], conducted on isolated and perfused lungs, proved a diversion of blood towards internal lung, since vasoconstriction of the subpleural region was not coupled by a reduction of total blood flow and arterial pressure increased during hypoxia treatment.

Relatively to capillary resistance, a study on isolated cat lungs during hypoxia administration found, by measuring vascular pressure by the micropipette method, a reduction of arterial and micro-vascular resistance, while venous resistance did not change [143]. It should be noted, however, that, at odds with ex-vivo isolated preparations, our closed-chest in-vivo preparation allows to observe functional adaptive response which cannot be captured by flow- and pressure-controlled experiments.

Differently from the in-vivo microscopic study on mice, showing a significant reduction of arteriolar but not of venular calibers [129], results from modelling suggest an increase of venular resistance, a factor having a possible important role in hypoxia-induced capillary recruitment in human lung. Venular and venous constriction have been found in rat lung and hypothesized in human lung and could be related to the hypoxic inhibition of potassium channels of the muscle cells forming venular sphincters [50]; from our results, this effect is evident for the short-term response (Phase I) and seems to attenuate for the more heterogeneous mid-term response (Phase II).

The present study provides also indications about the mid-term effect of acute hypoxic response; the PHASE II strong reperfusion pattern has not been documented by previous in-vivo microscopic studies, limiting their analysis to the first minutes after treatment [51] [129]. PHASE II reperfusion may be interpreted as an attempt to restore blood flow in those regions subject to vaso-constriction and to increased interstitial pressure, and should be considered an effect of the complex blood redistribution phenomenon. The
study of regional perfusion distribution through MRI arterial label spin sequence could help to investigate this aspect.

It should be noted that image-based modelling indicates that arteriolar and corner reperfusion is not coupled by septal recruitment; in fact, while in PHASE I de-recruitment is related to vasoconstriction, during PHASE II, de-recruitment is due to increased interstitial pressure reducing septal capillary patency. The hypothesis of corner recruitment in hypoxic conditions due to increased interstitial pressure, foreseen by the model presented in Chapter 3 and in [90] is thus confirmed.

Previous attempts to model pulmonary microperfusion patterns based on in-vivo microscopy were based on determining the randomness of capillary recruitment and found that capillary recruitment of single alveoli is independent from that the adjoining alveoli [144] [145]. The novelty of our image-based modelling approach lies in the possibility to estimate micro-vascular hemodynamic variables without measuring them; a validation study, based on micropuncture measurement of arteriolar, venular and interstitial pressure and possibly blood flow by laser reflectometry, will be required to assess the validity of the model. In fact, due to image resolution, our experimental approach did not allow to quantify red blood flows. These preliminary results are encouraging in that they provide the expected result of arteriolar vaso-constriction and suggest a possible important role for venular vasoconstriction in the early phase of interstitial edema.

5.5 Conclusions

1) The present study investigates the short-term and mid-term adaptative response of pulmonary micro-circulation and alveolar mechanics in the subpleural region in a closed-chest rabbit lung model of interstitial edema.
2) Short-term response of micro-vascular compartment in the subpleural region consists in a strong arteriolar vasoconstriction and a reduction of the network of capillary corners. Mid-term response is characterized by a recovery of lung perfusion and of capillary recruitment, even if in a condition of increased interstitial pressure, aiming to restore blood flow in subpleural regions.
3) Subpleural alveolar mechanics is affected by alterations of the interstitial lung fluid balance, revealing an interaction between structural and functional properties of the peripheral lung.
4) Applying the model to the experimental images suggests a possible role also for venular vasoconstriction. Furthermore, by integrating previous data on interstitial pressure for the mid-term period, results from modelling confirm that a strong corner reperfusion is not coupled by septal recruitment, due to increased extravascular water.
5) The model allows to estimate hemodynamic parameters from morphological measurements and to address several questions about the physiological mechanisms underlying adaptive response to hypoxia.
6. MRI quantification of lung edema

The content of this chapter will be submitted as part of an original research article to the *Journal of Applied Physiology*. 

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6.1 Introduction

Imaging pulmonary edema is challenging due to the rapid dynamic of edema onset, to the difficulty to distinguish among hydrostatic and permeability-induced edema and because of the small change of extra-vascular water in the early interstitial phase of the fluid accumulation. X-rays CT were used to measure local increase of extra-vascular water for example in re-expansion [146] or in exercise-induced [147] pulmonary edema, but suffers from invasiveness and does not provide indications about micro-vascular filtration rate. Micro-vascular barrier function can be obtained by scintillation detection probes revealing radioactivity of red blood cells labelled with $^{99m}$Tc [48], but this technique requires long scan times and has a limited resolution. Due to its intrinsic nature of measuring proton density, MRI can become a valuable non-invasive alternative to measure regional fluid accumulation in edemagenic conditions. The possibility of exploiting MRI different techniques to measure different parameters has put forwarded its use in research practice: beside lung fluid density, MRI has been used in the study of pulmonary edema to quantify pulmonary blood flow ([148], [149]), to assess the magnetic susceptibility related to the air/liquid interfaces related to the transverse relaxation time $T_2^*$ [150], or, by using hyperpolarized $^{129}$Xe, having a different chemical shift in blood and in interstitium, to evaluate interstitial septal thickening, considering the dynamics of Xe uptake ([151], [152]).

One major limitation of MRI images of lung parenchyma is the low signal-to-noise ratio (SNR), due to the low proton density of lung tissue, to the high heterogeneities of magnetic susceptibility at the air/tissue interface which reduces the exponential time of signal decay $T_2^*$ (0.5-3 ms [150]) and finally to respiratory and cardiac artefacts. Lung MRI imaging has improved by reducing the time echo (TE), i.e. the time intervening between the polarization pulse and the signal acquisition time, thanks to hardware advancements. This technique is named ultrashort time echo (UTE) and is based on submillisecond time echos and on a radial three-dimensional sampling of $k$-space. UTE has proven to be sensitive to change in density in human lung between end-expiration and end-inspiration and also to the gravity-dependent variation in proton density [153] [154]; furthermore, it has been validated as a sensible measure of water content in sponge phantoms, providing higher SNR with respect to fast gradient echo (GRE) [155], a technique already validated to estimate lung water content [156].

The aim of the present study is to study the sensitiveness of both GRE and UTE to measure small fluid accumulation in lung parenchyma and possibly to derive indications about the regional distribution of mild interstitial edema.
6.2 Methods

Experiments were performed in the Cincinnati Children's Hospital, in collaboration with the Center for Pulmonary Imaging Research (CPIR) group led by Prof. Jason Woods. Experimental protocol was approved by the Veterinary Service Children's Hospital Ethical Committee, in accordance with the IACUC guidelines. 40 C57BL6 mice, 8-13 weeks old, (weight range 16-30 g) were subdivided in four groups:

1) *group 1* (G1): 8 mice were immediately sacrificed to obtain control values of wet-to-dry ratio.

2) *group 2* (G2): 8 mice were imaged throughout 1 hour and a half, according to the timeline described in Figure 6.1, with no treatment except isofluorane anesthesia.

3) *group 3* (G3): 8 mice underwent 1 hour of hypoxic (10% O2) administration.

4) *group 4* (G4): 12 mice underwent a treatment of a single shot saline injection. Tail injection of 1ml of pre-heated saline solution was performed through a 30-gauge catheter inserted in tail vein before the imaging session.

Imaging procedures involved anesthesia administration to the animal by inhalation of 2% isofluorane mixture. Hypoxia and air lines were connected to the same three-way stopcock which output was directed to the isofluorane reservoir. We used a custom thoracic RF coil, within which the mouse laid supine; respiration was monitored by an accelerometer-based module and mouse body was heated and kept constant with a servo-controller module (Figure 6.2).

**Imaging procedures**

For groups 2, 3 and 4, the imaging protocol shown in Figure 6.1 was performed: each time point includes a Gradient-Echo (GRE) acquisition and a ultra-short time echo (UTE) acquisition. A complete time point requires about 20 minutes to be completed (about 5 minutes for GRE acquisition, and about 15 minutes for UTE). The imaging session involved three time points, lasting one hour and a half. For group 2, therefore, three acquisitions were obtained in control conditions, while for groups 3 and 4, one acquisition provided the baseline reference, while other two acquisitions were performed just after the treatment (time point identified as "0 post") and half an hour after treatment ("30 post").
**IMAGING PROTOCOL**

**G1 CONTROL**

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Baseline</th>
<th>0post</th>
<th>30post</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRE+UTE</td>
<td>GRE+UTE</td>
<td>GRE+UTE</td>
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</table>

**G2 ISOFLUORANE CONTROL**

<table>
<thead>
<tr>
<th>Time [min]</th>
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<th>30post</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRE+UTE</td>
<td>GRE+UTE</td>
<td>GRE+UTE</td>
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**G3 HYPOXIA**

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<tr>
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**G4 SALINE INFUSION**

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</tr>
</thead>
<tbody>
<tr>
<td>GRE+UTE</td>
<td>GRE+UTE</td>
<td>GRE+UTE</td>
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</tbody>
</table>

**Figure 6.1** Timing protocol for MRI imaging.

**GRE sequence**

Imaging parameters of GRE and UTE techniques are summarized in Table 6.1. Gradient-Echo sequence consists of a two-dimensional sequence based on the generation of rapid successive echoes by means of gradient switching. Respiratory gating was used to acquire data at end expiration. All slices were acquired for each k-space line during each respiration period. The repetition time (TR) is equal to the breathing period, while echo time was 0.749 ms. Image matrix = 96x96, FOV = 24mmx24mm, slice thickness = 1mm, slice gap = 0.1mm, number of slices 18. The number of repetitions for averages was 6, the flip angle used was 10°. The pulse sequence of GRE approach is shown in Figure 6.3, where "r" represents the read gradient, "p" is the dephase gradient and "s" is the slice selection gradient, having a refocusing inversion just after the RF pulse.
Figure 6.2 Experimental set-up for animal MRI imaging.

Figure 6.3 GRE pulse sequence.

**UTE sequence**

Self-gated UTE (with slab selected) was used. The repetition time was 4.9 ms, while the echo time was 0.628 ms. A total number of 147043 projections was used for each 3D acquisition, with around 50000 for expiration and 10000 for inspiration. The number of interleaves was 13; the imaged 3D volume is composed by 108x108x108 pixels and included a FOV of 30mmx30mmx30mm.
MRI quantification of lung edema

UTE pulse sequence is shown in Figure 6.4. Briefly, the radial acquisition sequence was implemented on the ParaVision 5.1 software platform (Bruker BioSpin MRIGmbH). Following the scheme proposed by [157], an excitation hard pulse precedes encoding gradients, which define the sampling trajectory of the $k$-space. The terminal points of each radial spoke define a spiral path on the surface of a sphere, providing a uniform sampling of the $k$-space. 3D volume was divided into 13 interleaved acquisitions, each of which is the rotated version of the preceding.

Figure 6.4 UTE pulse sequence.

<table>
<thead>
<tr>
<th>Parameter</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Repetition time (ms)</td>
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</tr>
<tr>
<td>Echo time (ms)</td>
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</tr>
<tr>
<td>Section thickness (mm)</td>
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<td>0.278</td>
</tr>
<tr>
<td>In-plane resolution (mm$^2$)</td>
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<tr>
<td>Flip angle (degrees)</td>
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<td>10</td>
</tr>
<tr>
<td>Respiratory gating</td>
<td>Prospective (respiration monitored by an accelerometer)</td>
<td>Retrospective (based on the phase of the FID, modulating with respiration)</td>
</tr>
</tbody>
</table>

Table 6.1 MRI GRE and UTE imaging parameters.

**Image analysis**

GRE and UTE volumes were analyzed slice by slice only along the along the caudo-cranial direction. A rod containing 1% Gadolinium saline solution was used to have a constant reference to normalize lung proton density values. Both right lung and left lung were segmented carefully, including only parenchyma and excluding vascular structure or lung consolidations due to atelectasis (Figure 6.5). Parenchymal proton density was normalized to the mean value of the rod intensity.
To compute the average increase in lung density, the following procedure was adopted for both GRE and UTE images: lung density normalized by rod was compared between each post-treatment time point and baseline slice by slice to obtain a percentage increase of lung density for each slice $\Delta \rho\%$. The percentage variation between post-treatment time points and baseline was averaged among all the slice to provide a mean value. The average was obtained by weighting the $\Delta \rho\%$ of each slice by the area of the corresponding segmented region of interest (ROI); from this analysis, an index, defined from this point onwards as $\overline{\Delta \rho}\%$, was obtained for both GRE and UTE.

As to UTE, since our custom UTE sequence had not been fully validated at the time of experiment, not all acquisitions were adequate to reliably assess lung density. Therefore, the quality of image was evaluated by considering the difference in density between the left and the right region of the reference rod (Figure 6.6). Only images having a value less than 20% of difference above the mean value were considered in the analysis, to avoid bias maybe due to incorrect image reconstruction filters.

**Figure 6.5** Representative GRE images of the same G4 mouse (M59) imaged before and after saline injection. Right and left lung, together with the reference rod containing a solution of 1% Gadolinium, were segmented, avoiding vessels and atelectatic areas.

Regional gradient of increase in lung density along the caudo-cranial direction was evaluated in GRE images for animals having an $\overline{\Delta \rho}\%$ greater than 30% at least at one time point and belonging to G3 or G4. For these animals, the regional distribution of $\Delta \rho\%$ was evaluated slice by slice along the caudo-cranial direction and normalized to the $\Delta \rho\%$ of the first slice close to carina.
Regional gravity-dependent increase of lung density was measured in UTE images by distinguishing lung density increase (slice by slice) between dependent and non-dependent regions of the lung, thanks to the higher resolution of this imaging technique compared to GRE (Figure 6.6). Gravity-dependent gradient was computed by dividing the normalized lung density of the gravity-dependent region by normalized lung density of the non-dependent region, slice per slice (along the caudo-cranial direction). The gradient was then averaged among slices (weighted by segmented ROI area) to obtain a mean value; finally, for post treatment time points, this average value was normalized to baseline.

Figure 6.6 Representative UTE images of the same G4 mouse (M59) imaged before and after saline injection. Right and left lung, together with the reference rod containing a solution of 1% Gadolinium, were segmented, avoiding vessels and atelectatic areas. Note that two regions of the reference rod were segmented to evaluate image uniformity and quality.

**Wet-to-dry ratio**

Immediately after the experiment, the animals were killed by a 5 minutes CO₂ administration. Thoracotomy was performed and lungs were carefully removed from the thoracic cavity; after having exsanguinated pulmonary arteries and veins, lungs were rapidly weighted. Lung tissues were dried in oven at 60°C for 60 hours and then their weight was estimated again to obtain a wet-to-dry ratio.
6.3 Results

Wet-to-dry ratio

Figure 6.7 shows the values of wet-to-dry ratio for all the groups: a 1-way ANOVA revealed a significant increase of lung water content only for saline injection group (Dunn's test, p<0.001) with respect to all the other experimental groups. A slight, even if not statistically significant increase is associated with the isofluorane group.

GRE

2-Way RM ANOVA was performed to assess the effect of the several treatments and of the time of treatment on lung density. Top row of Figure 6.8 shows the time pattern of $\Delta\rho\%$. Only for the saline group a statistically significant increase over baseline was found for both post-treatment timepoints. As to the effect of treatment compared within time point, saline injection $\Delta\rho\%$ was found to be greater than the hypoxic and isofluorane control group for "0 post" and greater only than the hypoxic group for "30 post".

![Graph showing wet-to-dry ratio for all groups.](image)

**Figure 6.7** Wet-to-dry ratio for all groups. * p<0.001, Dunn test, with respect to the other three treatments. G1 is the control group, G2 is the group subject only to isofluorane, G3 is the hypoxic group and G4 is the saline injection group.
Figure 6.8 $\Delta \rho\%$ obtained from GRE imaging. Top row represents the time pattern for each experimental group, bottom row shows the grouped data for each treatment at both treatment time point. †p<0.05 versus Baseline. *p<0.05 versus both G2 and G3. § p<0.05 G3.

Caudo-cranial distribution of edema

Five G4 mice overcame the threshold of 30% for $\Delta \rho\%$ at least at one time point. For these animals, $\Delta \rho\%$ values normalized to the $\Delta \rho\%$ of carina are shown slice per slice in the top row of

Figure 6.9. By grouping slices so as to distinguish three different zones along the caudo-cranial direction, a significant difference (p<0.05) between the apex region and the intermediate and bottom zones was found for both time points by a 2-way ANOVA (independent factors being time and lung region) (top row of

Figure 6.9). No statistical difference was found among time points.
Figure 6.9 Regional distribution of lung density increase normalized to carina $\Delta \rho \%_{\text{carina}}$ (*p<0.05 versus intermediate and bottom region).

UTE
Time patterns of $\overline{\Delta \rho} \%$ are shown in the top row of Figure 6.10, affected by a decimation of the database due to the described artefact correction. 2-Way RM ANOVA was performed to assess the effect of the several treatments and of the time of treatment on $\Delta \rho \%$. No significant increase for $\overline{\Delta \rho} \%$ was found in group G4, notwithstanding a tendency to follow the same trend of GRE results. Bottom row of Figure 6.10, gathering data for treatment exhibits an increase of G4 $\overline{\Delta \rho} \%$, even if not significant compared to the other treatment groups.
Figure 6.10 $\Delta \rho%$ obtained from UTE imaging. Top row represents the time pattern for each experimental group, bottom row shows the grouped data for each treatment at both treatment time point.

Figure 6.11 Average gradient among slices along the gravity dependent (antero-posterior), determined from UTE images. The gradient value is normalized to baseline. Top row shows the time pattern of normalized gradient, while the bottom row represents the gradient values grouped by treatment. *p<0.05 versus G3.
Gravity-dependent distribution of edema

Top row of Figure 6.11 shows the time pattern of the gravity-dependent gradient (dep/non dep density ratio, normalized to gradient at baseline). By grouping data per treatment (bottom row of Figure 6.11), a greater increase of gradient can be appreciated for the saline group. 2-way ANOVA performed on gradient increase, with independent factors being time and treatment, found a statistically significant (p<0.05) difference between G4 and G3 for both timepoints.

6.4 Discussion

This study investigates the sensitivity of two MRI imaging sequences to detect subtle variations of lung density, as occurring during the early phase of lung edema. To generate lung interstitial fluid accumulation, two different edemagenic treatments were used, namely hypoxia administration and saline injection. One-shot saline injection is a well-known edemagenic condition [158], since it solicits an increase of pulmonary blood flow and pressure. A 25-mg mouse total blood volume is about 2 ml [159], therefore a 1 ml injection increases blood volume of about 50%. Relative to hypoxia, even if previous experiments [85] revealed that evidences of interstitial edema and positive interstitial pressures during hypoxia administration are generated only after 2-3 hours, we chose to use the same 1-hour timing protocol for saline injection and for hypoxia administration to evaluate the sensitivity of MRI to different grades of pathology. 1-hour hypoxia was not found to alter lung fluid balance in mice. This result seems to confirm the hypothesis that short-term hypoxia is not able to generate positive interstitial pressures if it is not coupled by a significant increase of capillary pressure, for example at high altitude. This hypothesis, put forward by [48], is in line also with the results of our model presented in Chapter 3, where we reported data showing that hypoxia generated high $P_{\text{liq}}$ interstitial edema only after 6 hours. Our modelling interpreted the development of edema as generated by an increase of both lung capillary permeability and pressure (Figure 3.6).

Two control groups were considered, one immediately sacrificed without imaging procedures to obtain normal wet-to-dry ratio and one undergoing imaging procedure and anesthesia without other treatment. The reason lies in the potential edemagenic effect of anesthesia, already found in previous works studying the development of neurogenic pulmonary edema [160][161]; experimentally measured wet-to-dry ratios confirm the hypothesis of a potential interaction of anesthesia with the measured variable (lung density), even if the increase of lung density in G2 group compared to G1 is not significant.

Results of imaging prove that MRI GRE is able to detect and map the fluid accumulation for small changes of lung water content, occurred only for mice subject to saline
MRI quantification of lung edema

infusion; in fact, the wet-to-dry ratios of saline injection group lay in the range of mild interstitial edema and no evidence of alveolar edema were found by visual inspection of the extracted lungs, except for one mouse (M59), representing the outlier of the presented figures. To exclude the presence of small regional alveolar fluid accumulation, however, histological analysis would be required, since alveolar and interstitial edema cannot be distinguished based on the imaging. Custom UTE sequence was not able to reach statistical significance due to artefacts affecting some reconstructed images, thus reducing the available samples necessary for the required statistical power, but the trend confirms the one shown by GRE images.

It is worth noting that MRI allows also to obtain regional maps of lung fluid accumulation. In a saline slow-injection model of lung edema [9], a significant increase of endothelial layer was found in both the upper and lower region of the lung in rabbits, but only the latter showed an increase in the harmonic mean, representing the interstitial thickness in the thin portion of the air-blood barrier. Anyway, mouse model, chosen for its size compatibility with 7T MRI scanner and for the easiness of edema generation, is less subject to gravitational gradient, because of the reduced height on which this gradient can be exerted, so that that it does not exceed 1-2 cmH₂O.

A previous study demonstrated that oleic acid provides an increase in the bottom lung, probably caused by the greater blood flow in this region; interestingly, a reduced, even if not significantly, blood flow was found in these regions experiencing a greater increase in MRI proton signal, possibly related to vasoactive responses and/or mechanical compression of arterial distribution vessels [162]. A similar result was found in supine humans subject to rapid intravenous saline injection, causing an increase in perfusion only in the non-dependent part of the lung [163]. Notwithstanding the reduced gravitational gradient in mice, indications from the present study confirm the tendency of edema to concentrate in the gravity-dependent regions.

Relative to the non-gravity dependent direction, i.e. the caudo-cranial one, lung edema was found to concentrate in the apical region. One possible reason to explain this phenomenon is to consider that bottom and upper (apex) lung regions differ by capillary density, as proved by histological studies [9]. To interpret MRI results about regional distribution of lung edema, we may use the theoretical model described in [90] to provide a semi-quantitative evaluation of the behaviour of two lung ACUs, one with a low density (mimicking the behaviour of the upper lung) and one with a higher density (representing the bottom region). The networks are shown in Figure 6.12; the same planar extension and set of corner vessels and nodes was used for the two 3D ACUs, but density was modified accordingly to [9].
Figure 6.12 Two different ACU models, differing for capillary density for lung regions.

Figure 6.13 presents the results of modelling ACUs with different capillary density. Solid lines represent the low density ACU (upper lung or apex) while dashed lines describe the hemodynamic behaviour of the higher density ACU (bottom lung). Top panels show that both in normal condition, with subatmospheric interstitial pressure, and in edema conditions, low density ACU has higher recruitment values; furthermore, an increase of arteriolar pressure with elevated interstitial pressure produces a recruitment higher than the high density network. A higher degree of recruitment related to the total capacity of the ACU to accept blood flow means that, relative to extravascular water control, low density ACUs works at a higher balance point with respect to high density ACUs, having an ability to "store" more blood coming for example during a fluid overload or in exercise. Therefore, increased blood flow cannot be accommodated in unperfused capillaries and tends to increase pressure. This behaviour is confirmed also by the patterns of ACU resistance to blood flow: low density ACU preserves higher values compared to high density ACU; this aspect could play a role in the caudo-cranial gradient of fluid accumulation. In fact, if blood flow would increase in the same way for the bottom and the apex of the lung, due to the higher resistance, the low density ACU should experience higher capillary pressures, favouring filtration towards the interstitium.

Fluid filtration depends also on the available exchange surface area in static conditions; in dynamic conditions, however, an edemagenic stimulus will develop edema with a higher probability if the increase of surface area normalized to baseline is higher By
hypothesizing that, at baseline, $P_{art} = 16 \text{ cmH}_2\text{O}$ and $P_{liq} = -10 \text{ cmH}_2\text{O}$, and in interstitial edema condition $P_{art} = 20 \text{ cmH}_2\text{O}$ and $P_{liq} = 4 \text{ cmH}_2\text{O}$, assuming a constant $P_{ven} = 6 \text{ cmH}_2\text{O}$, both low density and high density ACU recruitments do not increase from baseline (Figure 6.13). Therefore, in these conditions, the main contribution to an increased filtration is related to increase of the Starling hydraulic gradient, greater in low-density ACUs with respect to high density ACUs.

More data are required in such a way that the model could fit the experimental results and predict the temporal pattern of lung edema. In conclusion, we think that theoretical modelling, corroborated by further experimental data from MRI sequence about not only extravascular water, but also lung perfusion and interstitial thickness (obtained from $^{129}$Xe imaging), may be used for exploring the overall lung response to perturbations of fluid balance, both in terms of regional and whole organ functional changes.
6.5 Conclusions

1) The present study investigates the sensitivity of MRI sequence to detect small changes in lung interstitial proton density and aims to evaluate the adaptative response of mouse lung to two different edemagenic conditions, namely hypoxia and saline injection.
2) A significant increase of the wet-to-dry ratio compared to control was found only for saline injection experimental group, probably related to a regional development of alveolar edema. If present, interstitial edema generated by hypoxia could not be revealed by an increase of the wet-to-dry ratio or of proton density detected by MRI sequences.
3) Lung density variation estimated by MRI imaging confirms the previous finding, thus indicating the possibility of MRI image to evaluate the early phase of lung edema.
4) For the saline injection group, both a crano-caudal and a gravity-dependent gradient were found. The latter can be explained by the small difference in hydraulic pressure along the vertical direction; the first may be due to the different capillary density of the upper lung compared to the bottom lung.
5) By comparing two ACU models differing by capillary density, it was found that the low density ACU displays a greater recruitment with increased arteriolar pressure, thus indicating a greater unbalance of lung fluid control during edemagenic conditions.
7. Conclusions
Pulmonary edema can be generated by several physiological and pathological concurrent mechanisms. Understanding the possible interactions among the variables involved in the fluid accumulation in pulmonary interstitium allows not only to obtain a deeper insight within the lung adaptive response to perturbative stimuli and within the pathophysiology of lung edema, but also to derive useful indications about possible treatments and preventive interventions for subjects prone to the development of this critical condition. In the present work, this mechanisms have been investigated in control and edemagenic conditions in animal models.

The aim of this Thesis was to develop new experimental, analytical and theoretical methods and models of pulmonary capillary fluid balance in order to elucidate the mechanisms of interaction between pulmonary microvasculature and interstitial mechanics in edemagenic conditions. The physiological variables acting in the control of lung extra-vascular water are the pulmonary capillary perfusion, the structural characteristic of the pulmonary interstitium and the lymphatic drainage. For this reason, we reviewed several modelling approaches for describing quantitatively both capillary perfusion and fluid balance and tried to incorporate them in a unique model able to capture the main mechanisms causing lung edema. In parallel with the theoretical analysis, we used two imaging techniques, namely in-vivo microscopy (IVM) and magnetic resonance imaging (MRI) to investigate alveolar structural alterations, perfusion patterns and lung density, which intervene in the control of fluid accumulation. Algorithms to segment alveolar borders and vessel caliber were presented and discussed together with the developed experimental setup required for IVM and MRI.

The first study introduced the theoretical model of the pulmonary microcirculation, based on previous formulations, but adapted to morphometric measurements for both animal and human lung; it was developed and validated against available data in literature about capillary perfusion. The modelling results relative to capillary recruitment were verified with data present in literature for the same species, interpolated and inserted within a model of extra-vascular lung water. By fitting experimental data with model simulations, it has been possible to identify the possible causes of increase of extra-vascular lung water either in capillary pressure increase or in capillary permeability, in two edemagenic conditions, namely hypoxia administration and collagenase injection, a treatment generating a destruction capillary barrier, thus increasing remarkably filtration rate. The model proved to be a useful tool for studying lung edema development.

In order to assess the possibility of electing in-vivo microscopy as the standard technique for the study of lung edema at the alveolar level, we analyzed subpleural alveolar mechanics in a rabbit model of healthy lung during maneuvers of inflation and deflation.
Conclusions

and evaluated alveolar specific and absolute compliance, estimated from a statistical method applied to alveolar area distributions. Results showed that absolute alveolar compliance is proportional to the baseline alveolar area; furthermore, the dependence of alveolar area on the topological distribution was examined by a clustering agglomerative algorithm. No evidence of topological dependence was found and scaling up from single alveoli to randomly selected alveolar regions, a relatively homogenous mechanical behavior with minimal hysteresis of overall alveolar expansion was observed. The results of the study suggest that the considerable heterogeneity of alveolar size and of the corresponding alveolar mechanical behavior are homogenously distributed, resulting in a substantially homogenous mechanical behavior of lung units and whole organ. Furthermore, IVM proved to be a proper technique for studying pulmonary microstructures.

For this reason, IVM was applied to study the vasoactive response of subpleural microvascular compartment to hypoxic administration. Two main temporal phases were identified. The first is characterized by a strong arteriolar vasoconstriction, a reduction of the caliber of corner vessels feeding septal network, greater at end-inspiration compared to end-expiration. In the second phase, a substantial re-perfusion of distribution vessels and of corner capillaries was observed. A significant alteration of alveolar mechanics characterized the whole experiment. In order to estimate the functional mechanical variables acting at the capillary micro-level for the control of alveolar perfusion, we applied the ACU model to the experimental images. A comparison between the vascular pressure estimated from the model and the changes of caliber of corner capillaries allowed to confirm the hypothesis of arteriolar vasoconstriction but suggested also an important role for venular vasoconstriction, a phenomenon which occurrence in edemagenic condition is still under debate. Thus, the model allows to estimate hemodynamic parameters from morphological measurements and to address several questions about the physiological mechanisms underlying adaptive response to hypoxia.

Finally, a MRI study on in-vivo mouse lung model was conducted to evaluate the regional distribution of fluid accumulation due to two different treatments providing subclinical interstitial edema, namely hypoxia administration and saline injection. Results shows a correspondence between wet-to-dry ratio and the proton density estimated by both the MRI sequence imaging used. Furthermore, a gradient of edema distribution in both the caudo-cranial and the gravity-dependent antero-posterior direction was found, with both the apical and the dependent regions showing a greater increase of proton density. This result was interpreted in terms of ACU model by comparing the behaviour of two ACUs differing by the capillary density. Compared with the high density ACU, representing the bottom lung, low density ACU, typical of the upper lung, displays a greater increase of capillary recruitment while capillary pressure raises, especially in condition of elevated interstitial pressure. A greater increase of surface area for filtration for low density ACU may be the cause for increased filtration.
and thus for the observed distribution of lung edema. We think that theoretical modelling, corroborated by further experimental data from MRI sequence about lung perfusion and interstitial thickness (obtained from $^{129}$Xe imaging) may be used for exploring the overall lung response to perturbations of fluid balance, both in terms of regional and whole organ functional changes.

In conclusion, the present work provides a framework for further investigations which should take into account all the mechanisms described in this Thesis. Several aspects should be elucidated; as to modelling, filtration should be evaluated also with the Michel-Weinbaum formulation [35], which assumes a reduced filtration for low capillary pressures. Furthermore, a more organ-oriented modelling approach is desirable, in order to couple the hemodynamics of the pulmonary vascular tree with the microvascular perfusion characteristics and to study the effect of blood redistribution, perfusion inhomogeneity and adjacency interdependence. New experiments should be performed in order to measure other hemodynamic parameters, such as pulmonary artery pressure, perfusion patterns in the internal parenchyma, blood-gas concentrations, lymphatic drainage, so as to feed the model with more experimental data. In conclusion, we foresee the application of this integrated approach, involving theoretical modelling and experimental verification/validation, to obtain an integrated view of the underlying physio-pathological mechanisms occurring in the development of lung edema in several disease conditions, ranging from exhaustive exercise to pathological conditions, including adult and neonatal respiratory distress syndromes.
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Chapter 8


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Journal publications


Conference abstract
