Aspects on ventilation induced stress and strain on regional and global inflammation in experimental acute respiratory distress syndrome

JAIME RETAMAL MONTES
Abstract

Mechanical ventilation (MV) is a life-saving therapy in acute respiratory distress syndrome (ARDS), a condition that affects 3000 patients/year in Sweden with a mortality rate of about 40%. However, MV may induce or worsen lung injury causing “ventilator-induced lung injury (VILI)”. From a mechanical perspective strain (deformation, or relative change in lung volume) and stress (tension) have been postulated as main determinants of VILI. High respiratory rate is potentially another factor that may exacerbate VILI by amplifying the total energy transmitted to the lungs during MV. In this thesis in animal ARDS models the hypotheses were that 1) lung parenchyma inhomogeneities concentrate stress and amplify lung damage and inflammation, 2) higher respiratory rates increase lung inflammation and lung edema in heterogeneous ARDS, and 3) local lung deformation is related to local inflammation.

First, in a rat model the effect on inflammation and structural damage of regional lung collapse on the healthy surrounding lung tissue was assessed. Second, in porcine models the effect of respiratory rate on lung edema and inflammation was studied during two ventilatory modes; a) a permissive collapse mode and b) a homogenized lung parenchyma mode. Finally, lung deformation was correlated with lung inflammation assessed by positron emission tomography using 18F-FDG uptake.

It was found that; 1) local inhomogeneities can act as stress amplifiers, increasing lung tissue inflammation and damage in the healthy surrounded lung. 2) high respiratory rate increases lung edema but decreases lung inflammation when permissive lung collapse is used and that these effects are prevented with lung parenchyma homogenization; 3) local lung deformation and inflammation are well correlated.

In conclusion, lung inhomogeneities may aggravate VILI, respiratory rate may affect in different ways VILI progression depending on the ventilatory strategy, and finally, lung deformation is closely related to lung inflammation. With the caveat that the studies are performed in animal models, the results suggest that using ventilator strategies that homogenize the lungs, i.e., open collapsed lung regions and prevent re-collapse in ARDS will reduce VILI and in the end may decrease morbidity and the high mortality in this condition.

Keywords: ARDS, VILI, respiratory rate, strain, PET

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To Jimena my love, friend, and helpmate; to our beloved daughters: Florencia, Esperanza, Amalia, Olivia, and to our little Santiago
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV Retamal, J., Hurtado, D., Villarroel, N., Bruhn A, Bugedo G., Amato, M., Costa, E., Hedenstierna, G., Larsson, A., Borges, JB. (2016) Regional pulmonary deformation is positively correlated with regional lung inflammation assessed by $^{18}$F-FDG positron emission tomography / computed tomography, (manuscript)

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Abbreviations

\(^{18}\)F-FDG \([^{18}\text{F}]\text{fluoro-2-deoxy-D-glucose}\)
AKT protein kinase B
ANOVA repeated-measures analysis of variance
AP arterial pressure
ARDS acute respiratory distress syndrome
ARDSNet ARDS Network
AT atelectasis
BAL bronchoalveolar lavage
bpm breaths per minute
CD dorsal/dependent region of the inferior lobe
Cdyn,rs dynamic compliance of the respiratory system
CI caudal intermediate region from the inferior lobe
CINC-1 cytokine-induced neutrophil chemoattractant
CO cardiac output
CT computed tomography
CV ventral/non dependent region from the inferior lobe
DAMP damage associated molecular patterns
EELV end-expiratory lung volume
ENAC \(\alpha\beta\gamma\) epithelial sodium channel complex
EOE end of expiration
EOI end of inspiration
EVLW extravascular lung water
FAK focal adhesion kinase
\(F_i O_2\) fraction of inspired oxygen
FRC functional residual capacity
HRR high respiratory rate
I:E ratio inspiratory to expiratory time ratio
ID inner diameter
IDS instrumental dead space
IL-10 interleukin-10
IL-1\(\beta\) interleukin-1 beta
IL-6 interleukin-6
IL-8 interleukin-8
IM middle lobe-intermediate
\(^{18}\)F-FDG Ki \([^{18}\text{F}]\text{fluoro-2-deoxy-D-glucose net uptake rate constant}\)
LPS lipopolysaccharide
LRM  lung recruitment maneuvers
LRR  low respiratory rate
OLA  Open lung approach
PaCO₂  partial pressure of carbon dioxide
PaO₂  partial pressure of oxygen
PaO₂/FI O₂ ratio of partial pressure of arterial oxygen to the fraction of inspired oxygen
PCV  pressure-controlled mode
PEEP  positive end expiratory pressure
Peri-AT peri-atelectasis
PET  positron emission tomography
R/D  recruitment – derecruitment
ROI  region of interest
RR  respiratory rate
SA  superior lobe-apical
SD  standard deviation
Smad2 mothers against decapentaplegic homolog 2
Smad3 mothers against decapentaplegic homolog 3
sVol change in gas volume during inspiration/end-expiratory gas volume
TGF-β transforming growth factor beta
TNF-α tumor necrosis factor alpha
V/Q ratio of ventilation to perfusion
V/S volume-to-surface ratio
VCV volume-controlled ventilation
VILI ventilator induced lung injury
VT tidal volume
W/D wet-to-dry ratio
ZEERP zero end-expiratory pressure
ΔP driving pressure (difference between end-inspiratory and end-expiratory pressure)
Introduction

Acute Respiratory Distress Syndrome

The acute respiratory distress syndrome (ARDS) is an acute, diffuse, edematous inflammation of lung tissue induced by increased permeability of the alveolar-capillary membrane. Clinically, ARDS is characterized by impaired oxygenation due to increased venous admixture, decreased lung compliance, increased physiological dead space, and bilateral radiographic opacities[1, 2]. The underlying cause of the syndrome is a reaction by the pulmonary parenchyma to a variety of serious conditions, the most frequent of which are sepsis, severe pneumonia, peritonitis, and multiple trauma[3]. According to the recently proposed Berlin definition ARDS can be divided into mild, moderate, and severe, depending on the PaO$_2$/FiO$_2$ values[2].

Before the Berlin definition, population-based estimates of the yearly incidence of moderate and severe ARDS ranged from 3 to 88 per 100,000 people. In critically ill patients hospitalized in intensive care units (ICUs), the prevalence of ARDS has been estimated to be about 5–15% of patients[4]. The overall mortality is 15–50% in all major series, although several randomized controlled trials, some including mild cases, have reported a better survival in selected ARDS patients[5, 6].

On gross pathological examination, the lungs of patients who have not survived ARDS are heavy due to atelectases, interstitial and alveolar edema, and hyaline membranes[6]. On examination with laser confocal imaging, ARDS lungs have both collapsed and overdistended alveoli with a range of air pockets of various sizes surrounded by fluid and foam[7].

Tissue damage during ARDS involves disruption of endothelial and epithelial surfaces, flooding of alveolar spaces, inactivated surfactant, and an inflammatory reaction. The lung inflammation in ARDS is initiated, amplified, and modulated by a complex network of cytokines and other proinflammatory mediators produced by a variety of cell types in the lungs, including fibroblasts, epithelial cells, and inflammatory cells[8].

With regard to lung function in ARDS, hypoxemia is the most prominent feature. It is due to extensive collapsed and poorly ventilated lung areas with shunts and ventilation-perfusion (V/Q) mismatches[9]. In addition, dead space is markedly increased, which increases ventilatory demands[10]. Alterations in lung mechanics include decreased compliance due to the significantly large proportion of the lungs that is functionally lost because of col-
lapse, consolidation, and flooding[11]. Thus, ventilation takes place in functionally small lungs (a condition termed “baby lung”) [12]. In addition, there is marked heterogeneity in regional aeration. However, bronchoalveolar lavage (BAL) studies indicate that even normally aerated areas are substantially inflamed[13].

Ventilation-induced lung injury

Although mechanical ventilation (MV) is the most important therapeutic support in ARDS patients, it may induce or worsen lung injury, a mechanism and condition termed “ventilator-induced lung injury (VILI)”[14, 15]. Several experimental studies have shown that MV may induce lung injury even in previously healthy lungs when high tidal volumes ($V_T$) or when high end-inspiratory plateau pressures are used[16]. However, when the lungs are afflicted with already established ARDS of whatever origin, they appear particularly sensitized to the injurious effects of MV, even with moderate $V_T$ (12 ml/kg)[15]. As in ARDS the lungs have fewer lung units for gas exchange and alveolar ventilation; a rather normal $V_T$ can induce overdistension. In addition, several lung units become unstable in ARDS, aerating during inspiration but collapsing again during expiration. Thus the lungs suffer from repetitive cycles of recruitment/derecruitment (R/D), which has been shown to induce further lung injury[17].

From a mechanical perspective, the forces applied by the MV to the lung may be expressed as stress (tension, which may correspond in the lungs to transpulmonary pressure) or strain (deformation, which may correspond to the relative increase in lung volume). Strain has been postulated as the main determinant of VILI. However, strain has a static or tonic component determined by positive end expiratory pressure (PEEP) and a dynamic or phasic component determined by $V_T$[18].

Mechanical forces applied to the lungs may induce a biologic response termed biotrauma, characterized by lung and systemic inflammation[19]. As nicely showed by Ranieri et al. in patients with ARDS, injurious MV increases pulmonary cytokines, but more importantly, MV also induces loss of compartmentalization of lung inflammation. This leads to systemic cytokine release, which in turn may cause multiple organ failure and death[20]. Imai et al. showed that damage to distant organs and organ failure secondary to injurious MV may not only involve inflammation, but also apoptosis[21]. In contrast, protective MV allows pulmonary and systemic cytokines to decrease, which may explain the favorable results obtained by use of protective ventilation observed in large trials. Therefore, biotrauma, manifested by cytokine release, appears as the critical link between VILI and death[19-22].
Stress, strain, and stress raisers

The structure responsible for bearing the mechanical stress of respiration and giving support to the endothelial and epithelial cells is a skeleton made up of elastin and fibrillar collagen[18]. Secondary to non-physiological deformation, cells can react by secreting cytokines and proinflammatory molecules that can initiate an inflammatory reaction. These cells can be activated through direct damage or by mechanotransduction signaling[23]. In addition, the fibroelastic skeleton can be disrupted and these fragments can work as Damage Activated Molecular Patterns (DAMP) and activate an inflammatory response via Toll-like receptors[18, 23, 24].

Lung distention is commonly inferred from $V_T$ (ml/kg of ideal body weight) and end-inspiratory (plateau) airway pressure[25]. However, neither of these two variables reliably reflects tissue deformation, especially during acute lung injury when relationships between body weight and functional residual capacity (FRC) and between airway and transpulmonary pressure become unpredictable[26, 27].

A practical way of approaching the real tissue deformation is by assessing the volumetric strain, understood as the ratio of change of volume and the resting lung volume ($\Delta V/V_0$). In this ratio, $\Delta V$ corresponds to $V_T$, and $V_0$ corresponds to the resting volume, usually estimated as the FRC volume[18, 27, 28]. Another way to approximate this concept is by the assessment of $sVol$ that correspond to the change in gas volume during inspiration divided by end-expiratory gas volume, the gas volumes is measured from CT images[29].

Recently, Protti and coworkers have shown that high strain levels were associated with ventilator-induced lung injury when it exceeded a threshold of 1.5 to 2.5. In addition, the same group showed that the dynamic strain is the main determinant of VILI[27, 28]. This approach to the volumetric strain is global, summarizing the mechanical behavior of whole lungs. However, at the microscale level, the situation is probably different, with multiple regional strains of different magnitudes.

When an alveolus collapses, the traction forces exerted on its walls by adjacent expanded units increase and become concentrated. These forces may promote re-expansion of the alveolus at the expense of potentially injurious stresses at the interface between the collapsed and the expanded units[30-32]. These inhomogeneities are also known as pressure multipliers or stress risers[18, 26, 31, 32]. This conceptual framework was described by Mead and coworkers in 1970 and is essentially related to alveolar interdependence phenomena[18, 32]. In their theoretical analysis, Mead et al. estimated that the alveolar pressure at the junction of the fully collapsed and expanded alveoli could be as high as 4 to 5 times the applied pressure[33]. This landmark estimation of Mead of approximately 4 times local amplification was recently confirmed using synchrotron-based X-ray tomographic microscopy.
in a rat lung preparation[34]. There is clinical information that supports this theoretical model. Cressoni and coworkers showed that inhomogeneities assessed by CT image analysis were associated with overall disease severity and mortality[34]. In addition, ventilatory techniques like higher PEEP[35-37] or the prone position[35, 38], decrease lung inhomogeneity and, consequently, reduce the extent of stress risers by keeping open previously collapsed regions. This in turn reduces the risk of VILI and may potentially improve survival of ARDS patients[39].

**Respiratory rate as a determinant of VILI**

The product of the transpulmonary pressure and the \( V_T \) changes relative to the end-expiratory lung volume (EELV) can be viewed as the energy delivered by the ventilation within the lungs and an estimate of the damage the ventilator can do to the lungs. In this framework, the development of VILI may be also relevantly modulated by the cyclic frequency of stress application[40-43].

There are no clinical data regarding RR and VILI; however, there are some experimental studies that have assessed this relationship. These studies have shown that a low respiratory rate protects the lungs, decreasing inflammation, edema, and alveolar damage. However, tidal volumes, inspiratory times, and inspiratory flows vary between the studies, and in most of the studies very extreme values of RR were used[40-42, 44-46]. Recently, in a porcine model, Cressoni et al., showed that VILI develops if a mechanical power threshold is exceeded. Mechanical power was defined as the function of transpulmonary pressure, tidal volume (\( V_T \)), and respiratory rate[41].

In vitro studies show that cyclical mechanical stretch on alveolar epithelial cells cultures increases vascular permeability, inflammatory cytokine production, apoptosis, and activates mechanotransduction pathways like AKT, FAK and TGF-\( \beta \)[47, 48].

**Respiratory rate and protective mechanical ventilation**

Clinical studies have demonstrated the importance of lung-protective ventilation strategies to minimize VILI and improve outcomes[25, 49-51]. The National Institutes of Heart, Lung and Blood ARDS Network low tidal volume (ARDSNet) trial showed that use of low tidal volume (\( V_T \)) ventilation (6 vs. 12 mL kg\(^{-1}\) predicted body weight) significantly reduced mortality[25]. This strategy is the current standard for mechanical ventilation of patients with ARDS[52, 53]. During the use of the ARDSNet strategy, a high respiratory rate (RR) may be necessary to maintain adequate carbon dioxide removal in order to achieve a pH of > 7.30[25, 54]. However, there
is no solid experimental data supporting the safety of a high RR with regard to VILI[42]. In fact, in clinical trials, RR is set according to pH values, auto-PEEP, or to an arbitrary maximal rate (i.e. 35 bpm)[25, 33, 50].

Lung-protective ventilation strategies do not avoid/prevent completely the occurrence of alveolar collapse, overdistension, and R/D phenomena[55]. In the scenario of inhomogeneous lung parenchyma, the stress and strain may be unevenly distributed, thereby inducing localized areas that are subjected to very high mechanical stress, as described from a theoretical background by Mead and coworkers[56].

Open lung approach (OLA) is a ventilatory strategy aimed mainly at reducing dynamic strain, recruiting (reopening) non-aerated areas using lung recruitment maneuvers (LRM), and subsequently keeping them open by applying adequate levels of PEEP[57-59]. OLA strategies have been shown be able to homogenize lung parenchyma[57], improve oxygenation, and decrease lung inflammation[58].
The aims of this doctoral thesis

The main objective of the studies included in this thesis was to investigate the effects of inhomogeneities and respiratory rate on the development of ventilator-induce lung injury.

The specific aims of the studies were as follows:

I To assess the effect of local non-lobar atelectasis on inflammation and structural alveolar injury in the surrounding healthy lung tissue during mechanical ventilation.

II To assess the effect of respiratory rate on lung inflammation and edema in an experimental model of acute respiratory distress syndrome with permissive alveolar collapse.

III To assess the effect of a strategy of open lung approach on the independent effect of respiratory rate on inflammation and lung edema in the acute respiratory distress syndrome.

IV To correlate local tidal deformation with regional lung inflammation by using a mathematical model of finite elements to re-analyzing images from a previous study in a porcine experimental model of acute respiratory distress syndrome.
Material and Methods

Animals

The study reported in paper I was approved by the Ethics Committee on the Use of Animals - Health Sciences Centre from the Federal University of Rio de Janeiro (IBCCF-188-05/16). All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society. Thirty-five male Wistar rats (250 to 340 g) were taken from nursery of the Instituto de Biofísica Carlos Chagas Filho - Rio de Janeiro/Brazil. Animals had free access to water and food until being anesthetized.

The studies reported in papers II, III, and IV were approved by the Animal Ethics Committee in Uppsala (C 5/14), and the animals were treated in accordance with the National Institute of Health’s guidelines (NIH). We studied 12 piglets in each study (2 to 3 months old, weighing 27–32 kg) of mixed Hampshire, Yorkshire, and Swedish country breeds. Animals had free access to water and food until being transported to the experimental facility.

Anesthesia

In paper I, animals were anesthetized intraperitoneally (IP) with 0.4 mg/kg of midazolam (Dormicum®, 5 mg·ml⁻¹, Roche, Grenzach-Wyhlen, Germany) and 60 mg/kg of ketamine (Ketavet®, 100 mg·ml⁻¹, Pfizer, Berlin, Germany). The injections were repeated at half the dose every 30 minutes during the experiment. The depth of anesthesia was monitored continually by observing arterial blood pressure and heart rate, and additional doses of anesthesia were administered to assure the wellbeing of the animals.

In the experiments reported in papers II and III, animals were premedicated by an IM injection of xylazine (2.2 mg kg⁻¹, Rompun®; Bayer, Leverkusen, Germany) and tiletamine/zolazepam (6 mg kg⁻¹, Zoletil®; Virbac, Carros, France). After tracheostomization and peripheral vein cannulation, a bolus of fentanyl 0.02 mg kg⁻¹ was given IV. Anesthesia was maintained with fentanyl 0.04 mg·kg⁻¹·h⁻¹, ketamine 30 mg·kg⁻¹·h⁻¹ (Ketavet®, 100 mg·ml⁻¹, Pfizer, Berlin, Germany), and midazolam 0.1 mg·kg⁻¹·h⁻¹ (Dormicum®, 5 mg·ml⁻¹, Roche, Grenzach-Wyhlen, Germany), and after checking that anesthesia was
sufficient to prevent responses to painful stimulation between the front toes, muscle relaxation was achieved by rocuronium 0.3 mg/kg/h (Esmeron®, 10 mg·ml⁻¹, MSD, NY, USA). The depth of anesthesia was monitored continually by observing arterial blood pressure and heart rate.

Animal instrumentation

In paper I, under anesthesia tracheotomy was performed under anesthesia with a snugly fitting cannula (1.5 mm ID). An arterial catheter (18 gauge × 8 cm, Arrow International, USA) was inserted into the right carotid artery for continuous arterial pressure (AP) monitoring and for blood sampling. At the end of surgical instrumentation, animals were paralyzed (pancuronium bromide, 0.3 mg/kg, intravenously (IV)) and mechanically ventilated (Inspira ASV, Harvard Apparatus, Holliston, MA, USA) using volume-controlled ventilation (VCV) mode, tidal volume ($V_T$) of 6 ml/kg, respiratory rate (RR) of 90 breaths/minute, inspiratory to expiratory time ratio (I:E) of 1:2, positive end-expiratory pressure (PEEP) of 0 cmH₂O and oxygen inspired fraction ($FIO_2$) of 1.0, which were the baseline settings.

In papers II and III, at baseline, the lungs were ventilated with volume-controlled mode with a $V_T$ of 8 ml/kg, RR 30 breaths per minute (bpm), PEEP 5 cm H₂O, I:E 1:2, $FIO_2$ = 1. In paper IV, baseline ventilation was delivered in pressure-controlled mode (PCV) with a driving pressure of 12 cm H₂O, respiratory rate (RR) to keep PaCO₂ between 35 and 45 mmHg, positive end-expiratory pressure (PEEP) 5 cm H₂O, inspiratory to expiratory ratio (I:E) 1:2, and $FIO_2$ of 1.0.

The protocols regarding hemodynamics and monitoring were similar in papers II, III and IV. Thirty ml/kg/h of Ringer’s acetate was infused IV during the first hour. From the second hour to the end of the establishment of the 2-hit injury VILI model (see below), Ringer’s acetate infusion rate was 20 ml/kg/h. Thereafter, the infusion rate was kept at 10 ml/kg/h till the end of the experiment. After open dissection of the neck vessels, a central venous catheter was inserted via the right external jugular vein. A pulmonary arterial catheter (Edwards Life-Sciences, Irvine, CA, USA) for measurement of cardiac output (CO) and monitoring of pulmonary artery pressure was introduced via the right external jugular vein. Invasive systemic arterial pressure was monitored through a femoral arterial catheter (PV2015L20, Pulsion, Munich, Germany). ECG electrodes were applied on the chest and a pulse oximeter on the base of the tail for continuous monitoring. A lower midline mini-laparotomy was performed, and a bladder catheter was inserted to measure hourly urine production. After the instrumentation, a period of stabilization of 15 min was implemented. Volumetric capnography was performed with a NICO monitor (Philips, Wallingford, CT, USA) using airway flow and CO2 signals from mainstream sensors placed between the endotra-
cheal tube and the “Y” piece of the ventilator circuit. Data were recorded by the Datacoll software (Philips) and later handled by the Flowtool software (Philips). Calculations of respiratory mechanics were based on NICO monitor airway pressure and flow waveforms. Gas exchange measurements were made using conventional blood gas analysis (Radiometer ABL 505; Radiometer OSM3), and derived parameters were calculated according to the standard formula.

Atelectasis induction
In paper I, atelectasis was induced by bronchial blocking: after 5 minutes of stabilization and pre-oxygenation with $F_{I\text{O}_2} = 1$, a silicon cylinder blocker (diameter 1.9 Fr, and length 1.5 mm) was attached to a catheter metallic guidewire (L-Cath, Becton Dickinson, NJ, USA) and inserted through the tracheostomy until wedged in the terminal bronchial tree, where it was released through displacement of the catheter over the guidewire, softly pulling the silicon piece against the lung.

ARDS model
In papers II and III, we used the same ARDS model. After induction of anesthesia, instrumentation and physiological baseline measurements that included arterial blood gases, hemodynamics, and respiratory parameters, we established a 2-hit injury ARDS model: repeated lung lavages with 30 ml/kg of warmed isotonic saline were applied until a pressure of arterial oxygen to fractional inspired oxygen concentration ($PaO_2/F_{I\text{O}_2}$) less than 27 kPa was achieved. This was followed by 120 min of injurious mechanical ventilation using low PEEP (mean PEEP = 2 cmH$_2$O), high inspiratory pressures (mean plateau pressure = 36 cmH$_2$O), RR 20 bpm, and I:E 1:2. This model has previously been found to produce inflammatory changes compatible with ARDS.

In paper IV, after physiological baseline measurements, we established a 2-hit ARDS model: repeated lung lavages with 30 ml/kg of warmed isotonic saline were applied until a $PaO_2/F_{I\text{O}_2}$ less than 27 kPa was reached, followed by 210 minutes of injurious mechanical ventilation using low PEEP (mean PEEP = 4 cm H$_2$O), high inspiratory pressures (mean plateau pressure = 45 cm H$_2$O), RR 20, and I:E 1:2. At the end of this period, we recorded a new set of physiological data. After establishment of the ARDS model, ventilation was delivered in the PCV mode with a plateau pressure of 42 cm H$_2$O, RR 20, I:E 1:1, and $F_{I\text{O}_2}$ of 1. PEEP was applied at the minimal level to keep oxygen saturation obtained by pulse oximetry over 90%. These settings were maintained for 4 hours until the PET/CT study. Any glucose-containing IV
infusion was stopped 6 hours before the PET study. Fluid therapy, using Ringer’s acetate 5 ml/kg/h, was maintained at a constant level throughout the study period.

**Mechanical ventilation protocol**

In paper I, the animals were randomized after bronchial blocking to one of two groups according to the following mechanical ventilation settings: a) $V_T = 10$ ml/kg and PEEP = 3 cmH$_2$O ($V_T10$/PEEP3); and b) $V_T = 20$ ml/kg and PEEP = 0 cmH$_2$O ($V_T20$/zero end-expiratory pressure (ZEEP)). We chose these settings to evaluate the injury modulation using a clearly injurious modality ($V_T20$) and a less injurious $V_T$ setting ($V_T10$). In addition, in the last group we applied PEEP to maintain similar levels of mean airway pressure in both groups. The respiratory rate was 30 breaths per minute, I:E was 1:2, and $F_{I\_O_2} = 0.5$. Six animals in each group were mechanically ventilated for 180 minutes. Instrumental dead space was increased in the $V_T20$/ZEEP group to keep a similar arterial partial pressure of carbon dioxide (PaCO$_2$) levels between groups.

In paper II, we applied the ARDSNet strategy. During the first 15 min of the application of the ARDSNet strategy, the following settings were used in all animals: $V_T$ 6 ml/kg, PEEP 14 cmH$_2$O, inspiratory time 0.5 s, $F_{I\_O_2} = 0.7$, and RR 30 bpm. At the end of this period, the H0 measurements were performed. Then, the animals were randomized to RR = 40 bpm (high respiratory rate group; HRR) or RR = 20 bpm (low respiratory rate group; LRR). To keep similar PaCO$_2$ levels between groups and based on pilot data, we added adjustable instrumental dead space (IDS) in the HRR and cut the endotracheal tube in the LRR. The IDS was inserted between the “Y” piece and the distal flow sensor, which was placed at the endotracheal tube opening. This device allowed us to adjust the amount of rebreathed gas (that contained CO$_2$) to keep PaCO$_2$ at the desired level. Then, the following mechanical ventilation settings were applied and maintained until the end of the study: $V_T$ of 6 ml/kg, PEEP 10 cmH$_2$O, and $F_{I\_O_2} = 0.5$.

In paper III, once the ARDS model was established, a lung recruitment maneuver (LRM) was performed and followed by a decremental PEEP titration. The LRM settings were pressure-controlled ventilation, RR 20 bpm, I:E 1:1, and driving pressure 15 cmH$_2$O. PEEP was increased in 5 cmH$_2$O steps, over 20 s each step, from 10 to 40 cmH$_2$O. The final step was kept for 120 s. All animals underwent decremental PEEP titration. Starting from 25 cmH$_2$O, PEEP was decreased in 2 cmH$_2$O steps and maintained at that level for 3 min, before being again reduced by 2 cmH$_2$O. This continued down to a PEEP of 10 cmH$_2$O. Throughout the PEEP trial, $V_T$ was kept at 6 ml/kg. Dynamic compliance of the respiratory system (Cdyn,rs) was measured continuously and values were taken at the end of each PEEP step. After the end
of the decremental PEEP trial, the LRM was repeated, and the PEEP was set according to the PEEP, corresponding to the maximal Cdyn,rs with 2 cmH2O (called "optimum PEEP"). This procedure has produced full lung recruitment in our previous studies, as assessed by computed tomography scan. Then, the MV settings were as follows: volume-controlled ventilation, VT 6 ml/kg, optimum PEEP as defined above, inspiratory time 0.5 s, FIO2 1, and RR 30 bpm. After 15 min, the first measurements (T0) were taken. Thereafter, FIO2 was decreased to 0.5, and RR was either 40 bpm (HRR) or 20 bpm (LRR) according to a randomization procedure, with VT 6 ml/kg, and inspiratory time of 0.5 s in both groups. It is known that the PaCO2 level affects the immune response in experimental ARDS. Therefore, to keep similar PaCO2 (and VT) in the two groups, rebreathing was increased in the HRR group by adding an instrumental dead space between the Y-piece and the endotracheal tube. The settings were maintained unchanged until the end of the study.

Investigational Protocol

In paper I, gas exchange at baseline, lung mechanics, and hemodynamic parameters were assessed continuously during the whole protocol. Three series of experiments were performed, for histological analysis (n = 12), for tissue cytokines analysis (n = 12), and for micro-computed tomography imaging (n = 2). Also, another six nonventilated healthy animals were used as controls in the cytokines and histological studies.

In paper II, gas exchange, lung mechanics, and hemodynamic parameters were assessed after the initial stabilization period (Baseline), after the establishment of lung injury model (H0), and hourly during 6 h of application of the ARDSNet strategy (H1–H6). Plasma samples for cytokines measurements were taken at Baseline, H0, H3, and H6. Post-mortem, regional lungs samples were extracted to evaluate tissue cytokines, TGF-β pathway biomarkers, wet/dry weight ratio, and for histological analysis. Bronchoalveolar lavage fluid (BAL) samples were collected at the end of the protocol.

In paper III, gas exchange, lung mechanics, and hemodynamic parameters were assessed after the initial stabilization period (Baseline), after the establishment of lung injury (H0), and hourly thereafter during a total of 6 h of ventilation (H1–H6). Post-mortem, regional lungs samples were extracted to evaluate tissue cytokines, wet/dry weight ratio, and for histological analysis.

In paper IV, some of the data have been reported previously [60]. The Animal Ethics Committee of Uppsala University approved the study. We studied 12 piglets (2 to 3 months old, weighing 25.4 ± 3.5 kg) of mixed Hampshire, Yorkshire, and Swedish country breeds. All animals received IV anesthesia in the supine position using a combination of fentanyl, ketamine, and midazolam, as well as pancuronium for muscle relaxation, and were monitored as previously described [60]. To obtain control data, the same
imaging protocol and data analysis were performed in five animals, which did not receive saline lavages, and which were ventilated in a protective setting (tidal volume = 6 ml/kg and PEEP 5 cm H$_2$O) for 4 hours before the PET imaging.

Lung tissue sampling and histopathology

In paper I, at the end of the experiment, the animals were heparinized and exsanguinated by severing large abdominal vessels. The trachea was clamped at end-expiration and the lungs were extracted in block, fixed in formalin, and subsequently embedded in paraffin. In all animals, we performed the same procedure: the trachea was clamped at the end of the expiration, and all lungs were securely ligated at the level of main bronchi at this same pressure. Transversal slices from the apex to the base were cut (4-µm thick), carefully making sure to pass through the atelectasis. After repeating the same procedure in the contralateral lung without atelectasis, slices were stained with hematoxylin-eosin. Airspace injury was evaluated in a blinded fashion by a semiquantitative method that measured alveolar disruption, neutrophilic infiltration, edema, and hemorrhage, as previously described.[61, 62] Each of these variables was scored from 0 to 3 points according to the severity of the changes (0 = none, 1 = mild, 2 = moderate, and 3 = severe). First, we defined the following regions of interest (ROIs): atelectasis (AT) was defined as the alveolar collapse region (confirmed by direct observation of the silicon cylinder blocker during the tissue sampling); peri-atelectasis (PeriAT) was defined as the tissue portion 3 mm adjacent to AT, and control was defined as the anterior portion of the inferior lobe of the contralateral lung (Figure 1). Two slices from each lung were analyzed, randomly observing 10 fields (400X magnification) of each ROI. The scoring system was validated by review of selected sections together with a veterinary pathologist.

In papers II and III, during the opening of the chest wall after the animals had been euthanized, ventilation was maintained according to the protocol. Then, the inferior cava vein was sectioned, the trachea was clamped at end-expiratory lung volume, and heart and lungs were excised in bloc. Right lungs were clamped at the hilum, and lung tissue samples were collected from the following regions: superior lobe-apical (SA), middle lobe-intermediate (IM); and ventral/non dependent (CV), intermediate (CI), and dorsal/dependent (CD) regions of the inferior lobe. Samples were immersed in 10% buffered formalin, processed, and stained with hematoxylin–eosin for histological analysis. The CV, CI, and CD samples were evaluated histologically by a pathologist blindly. The pathologist performed a semiquantitative analysis based on alveolar collapse, emphysema, alveolar edema, alveolar and alveolar septa leukocytes infiltration, perivascular, bronchiolar,
peribronchiolar leukocytes infiltration, and interlobular and alveolar septa edema and leukocytes infiltration. Each item was scored based on the following scale: 0 = not observed; 1 = mild; 2 = moderate; 3 = severe; and 4 = very severe. Other samples in the same regions were snap-frozen in liquid nitrogen, kept at \( < 80^\circ \text{C} \), and later processed for biochemical and molecular biology analysis.

**Lung morphometry**

To determine the degree of inflation of the alveoli from the PeriAT and control regions, we used the volume-to-surface (V/S) ratio, a morphometric technique described by Weibel et al.[63]. A microscope field was projected onto a screen and a grid superimposed on the field. The grid consisted of 21 test lines with a length equivalent to 100 \( \mu \text{m} \) at the magnification used. The relative volume of the lung occupied by alveoli is equal to the percentage of points falling within alveoli (alveolar hits). The surface area of the alveoli is proportional to the number of times that test lines intersect the alveolar walls, and inversely proportional to the length of the line. The V/S ratio of the alveoli may then be calculated as follows:[64]

\[
V/S = \frac{\text{length of test lines} \times \text{times of alveolar hits}}{4 \times \text{times alveolar intersections}}
\]

**Wet-to-dry ratio**

Wet-to-dry (W/D) ratio was measured in samples of the same lung regions from the right lung. Briefly, samples of 2–4 g were weighed, dried in an oven at 50\(^{\circ}\)C for 72 h, and then weighed again.

**Bronchoalveolar lavage**

BAL was performed in the left lung by intrabronchial injection of 50 ml normal saline solution and subsequent aspiration. The recovered fluid was centrifuged and snap frozen.

**TGF-\( \beta \) pathway assessment**

Cryosections (5 \( \mu \text{m} \)) of formalin-fixed biopsies from pig lungs were stained with 2 \( \mu \text{g/ml} \) of rabbit polyclonal anti-phospho-Smad2 IgG.[65] Secondary goat anti-rabbit antibodies conjugated with Alexa Fluor 568 (Life Technologies) were used at 1:1000 dilutions. Nuclei were visualized with 1 \( \mu \text{g/ml} \)}
Hoechst 33342 (VWR International, Radnor, PA, USA). Fluoromount-G mounting medium (Southern Biotech, Birmingham, AL, USA) was used to mount stained sections. Microphotographs were taken with a Nikon Eclipse 90i microscope, using the 20X objective at the same exposure time for all samples.

Cytokines

In paper I, tissue samples from the three ROIs (AT, PeriAT, and control lung) were collected at the end of the ventilation protocols. They were identified and cut under direct vision, and immediately frozen and homogenized. Quantification of IL-1β and cytokine-induced neutrophil chemoattractant (CINC-1) was performed using ELISA with high sensitivity kits (R&D Systems Inc, Minneapolis, MN, USA) in accordance with the manufacturer’s instructions.

In papers II and III, the following cytokines concentrations were measured using the ELISA method in lung homogenates, plasma, and bronchoalveolar lavage (BAL): interleukin (IL) 1β, IL-6, IL-8, IL-10, and tumor necrosis factor alpha (TNF-α).

Micro-computed tomography (microCT)

To better characterize the atelectasis, we acquired two series of microCT images in two animals. One was made after 30 minutes and the other one 3 hours after induction of atelectasis. The microCT protocol was performed with 75 kV and 145 µA, 1.5 magnification, and three frames of 1,024 projections. The reconstruction was done with a body filter and gave a volume with a slice separation of 0.48 mm, 1024 X 1024 matrix, and recon voxel size of 0.170 mm.

Image Acquisition

We used a GE Discovery STE (GE Medical Systems, Milwaukee, WI, USA) PET/CT with a 64-slice Lightspeed CT. The PET and CT scan protocols were done according to previous description.[60] The section of the thorax to be imaged was selected on the scout view just above the diaphragm. A spiral CT scan of the chosen section was obtained while holding the animal apneic (by switching the ventilator to continuous positive airway pressure mode) at the same mean airway pressure as during mechanical ventilation to ensure the best possible cross-registration between the CT scan and the PET acquisition, performed during tidal ventilation. The animal was then ad-
vanced to the PET detector; the tomography ensured the cross-registration of the same axial field-of-view (15.3 cm) between the CT and the PET acquisition. $^{18}$F-FDG (~ 150 MBq) was infused and sequential PET frames were acquired while pulmonary arterial blood was sampled repeatedly. Activity in the plasma was measured in a gamma counter cross-calibrated with the PET scanner. The plasma activities of these samples were used to calibrate the blood-pool ROI and to obtain an image-derived input function taking into account partial volume and spillover effects [66]. In addition, we acquired CT scans at the end of expiration (EOE) and at the end of inspiration (EOI) by using the constant positive airway pressure mode. The duration of the CT scan acquisitions was around 3 seconds.

PET/CT Image Analysis

Images were analyzed using a customized program written in Lab-VIEW 7.1 (National Instruments, Austin, TX, USA) [60]. First, lung fields ROIs were manually outlined on the CT images, carefully avoiding the large airways, vessels, and pleural effusions. $^{18}$F-FDG net uptake rate (Ki) was calculated at the ROI level by fitting the $^{18}$F-FDG kinetics using the two-compartment model of Patlak et al.[67, 68]. Finally, we divided the 3D lung masks into 10 vertically distributed ROIs of equal height from top (ventral) to bottom (dorsal).

Regional Biomechanical Analysis

In order to estimate the regional values of volumetric strain and obtain 3D maps, CT lung images were analyzed following the image-based biomechanical analysis method described by Hurtado et al.[69] which will be briefly summarize in the following. Every biomechanical analysis performed considered a subsequent pair of EOE and EOI images. For each pair of CT images acquired, a non-rigid image registration was performed using the NiftyReg package [70]. The registration process delivered the transformation mapping that best aligns the EOE and EOI images by allowing a regional transformation of the EOE image. Then, by analyzing local changes of the transformation mapping, one can estimate regional measures of deformation. In particular, the regional volumetric strain could be directly obtained by direct differentiation of the transformation mapping, and computing the determinant of the Jacobian matrix. However, it has recently been shown [69] that direct differentiation leads to large errors in accuracy in the estimation of regional volumetric strain, particularly at the pleural interface. Therefore, to achieve higher accuracy in the estimation of the 3D volumetric strain map, a smoothing finite-element method was employed for the estimation of the
regional volumetric strain. First, the lung 3D domain was segmented out of an EOE lung CT image, from which a tetrahedral mesh was generated. Then, a variational projection that yielded the best orthogonal approximation of the strain tensor field onto the finite-element space was performed, which yielded a continuous field 3D representation of the regional volumetric strain. Finally, the value of regional volumetric strain was evaluated on the EOI image of the lung analyzed.

To be able to compare the regional volumetric strain image with the inflammation image of Ki, we computed average values of the volumetric strain for the ROIs as defined in the PET/CT image analysis section. To this end, we performed one additional image registration between the EOI and the mean airway pressure CT image, resulting in a transformation function that allowed mapping the volumetric strain image into this mean pressure configuration. Then, average values of the remapped volumetric strain were obtained for the same ROIs used in the PET/CT image analysis.

Spatial correlation between $^{18}$F-FDG and regional volumetric strain images

Based on the average Ki and volumetric strain ROI values described in the previous section, we constructed scatterplots of Ki versus volumetric strain, and then performed a linear regression. The correlation coefficient and p-value were computed for all subjects analyzed.

Statistical analysis

In paper I, variables were tested for normality with the Shapiro–Wilk test. For normally distributed variables, we used repeated-measures analysis of variance (ANOVA) for the comparison of any variable collected multiple times during the protocol. The Bonferroni adjustment for multiple tests was applied for post hoc comparisons. When the assumption of normality was not met, we used the Friedman test as the non-parametric alternative to the repeated measures ANOVA. Pair-wise planned contrasts were performed using the Bonferroni correction for multiple comparisons. To compare non-paired samples we used the Kruskal–Wallis test. All statistical tests were two-tailed, and the significance was set at $P < 0.05$.

In paper II, we assessed that for a $P < 0.05$, with a power of 0.8, six animals in each group were needed if the difference between the outcome variable W/D would be $1 \pm 0.5$. We expressed values as means ± standard deviation (SD) or median ± range where appropriate. The Shapiro–Wilk test was used to test data for normality. Groups were compared using Student’s t-test.
or the Mann–Whitney $U$-test, 1-way (repeated-measures) analysis of variance (ANOVA) or the Kruskal–Wallis test. Interactions between groups and time were assessed with 2-way repeated-measures ANOVA. The Bonferroni adjustment for multiple tests was applied for post hoc comparisons. The statistical analyses were conducted by SPSS v.20.0.0 software (SPSS, Inc, Chicago, IL, USA), and Graph-Pad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Statistical tests were carried out with the significance level set at $P < 0.05$.

In paper II, on the basis of previous data about the effect of respiratory rate on W/D ratio, we calculated that six animals per group were required in this study to observe a difference between groups of $1 \pm 0.5$ in W/D ratio, with a power of 0.8 and a $P$-value <0.05. We expressed values as means ± standard deviation (SD) or median ± range where appropriate. The Shapiro–Wilk test was used to test data for normality. Groups were compared using Student’s $t$-test, the Mann–Whitney test, 1-way (repeated-measures) analysis of variance (ANOVA), or the Kruskal–Wallis test. Interactions between groups and time were assessed with 2-way repeated-measures ANOVA. The Bonferroni adjustment for multiple tests was applied for post hoc comparisons. Statistical analysis was conducted with SPSS (version 20.0.0) and Prism GraphPad (GraphPad Software, San Diego, CA, USA). A $P$-value < 0.05 was considered statistically significant.

In paper IV, normality of the data was confirmed with the Shapiro–Wilk test. We expressed values as mean ± SD. We used the independent samples $t$-test for comparisons between groups and paired $t$-tests for comparisons within groups. $K_i$ was compared with a linear mixed-effect model, with group and region modeled as fixed effects and random intercepts for the subjects (lme4 package, R statistical environment, R 3.0.0, Vienna, Austria). Post hoc tests were adjusted using the Bonferroni correction. All tests were 2-tailed, with significance set at $P$ value of less than 0.05. Pearson’s correlation coefficient was determined to evaluate the relationship between radiotracer uptake and volumetric strain data (Prism 4.03, GraphPad Software, USA).
Results

Paper I

Both ventilatory strategies presented hemodynamic stability, and all animals survived until the end of the experiment. We choose the ventilator group settings to evaluate the injury modulation using a clearly injurious modality \( V_T 20 \) and a less injurious \( V_T \) setting \( (V_T 10) \), but the \( V_T 10 \) group resulted in a mean airway pressure 25\% higher and in a respiratory system elastance 33\% higher than the \( V_T 20 \) group. Also, the stress index was >1.2 in the \( V_T 10 \) group. Atelectasis was successfully induced in the basal region of the lung in almost all animals (26/29 animals). The microCT images of the animals revealed that the volume of the atelectasis was 0.12 and 0.21 cm\(^3\). The microCT images demonstrated that there was no lung collapse in any other areas at the start of the protocol. (Figure 1)

![Figure 1. Micro-computed tomography (MicroCT) images. MicroCT images are exhibited over time in one representative animal using the tidal volume = 20 ml/kg and positive end-expiratory pressure = 0 cmH\(_2\)O group. The black arrow indicates the atelectasis and the blocker.](image-url)
Atelectasis was evident in the lung samples of all animals analyzed and was consistent with the ex-vivo macroscopic confirmation. The microscopic analysis of the region inside the atelectasis did not show any alterations suggestive of alveolar disruption; however, it showed a large amount of intravascular neutrophils and vascular congestion. (Figure 2)

Figure 2. Histological and ex-vivo images. (A) Histological images (50X) of the atelectasis (AT) and peri-atelectasis (PeriAT) regions are exhibited. Note the alveolar hyperinflation of the surrounding alveoli. (B) Ex-vivo image of the lungs exhibiting the AT, PeriAT, and the control regions of interest. (C) Histological image of the contralateral lung (control region) of the same animal is exhibited. Note the difference in surface area of alveoli when compared with the PeriAT region.

Alveolar disruption and neutrophilic infiltration were higher in the PeriAT region than the corresponding contralateral lung control (Table 1). Edema was greater in the PeriAT region compared to the control in the $V_T 20$ group, but did not reach statistical significance in the $V_T 10$ group ($P = 0.06$). Contrasting the same corresponding ROIs, there were no greater differences between the $V_T 10$/PEEP3 and $V_T 20$/ZEEP groups (Table 1).

<table>
<thead>
<tr>
<th>$V_T 20$/ZEEP</th>
<th>periAT</th>
<th>control</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveolar disruption</td>
<td>1.3 (0.7–2.2)</td>
<td>1 (0.6–1.2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Neutrophils infiltration</td>
<td>3 (2–3)</td>
<td>1 (1–3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Interstitial edema</td>
<td>2.2 (1.4–2.6)</td>
<td>1.1 (0.9–1.2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>1.5 (0.9–2)</td>
<td>1.1 (1–2)</td>
<td>0.6</td>
</tr>
<tr>
<td>$V_T 10$/PEEP3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alveolar disruption</td>
<td>1.7 (1–2.2)</td>
<td>1.1 (0.9–1.2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Neutrophils infiltration</td>
<td>3 (2–3)</td>
<td>2 (1–2)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Interstitial edema</td>
<td>2.4 (1.7–2.7)</td>
<td>1.1 (0.8–2.1)</td>
<td>0.06</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>1.2 (1.1–2)</td>
<td>1.7 (0.8–2)</td>
<td>0.6</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alveolar disruption</td>
<td>1.4 (0.7–2.2)</td>
<td>1 (0.6–1.2)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
The V/S ratio was higher in the PeriAT region than the corresponding contralateral lung (control), demonstrating that the PeriAT region \((P < 0.05)\) presented more distension of alveoli. There were no differences in V/S ratio in the PeriAT region between the \(V_T = 10/\text{PEEP} = 3\) cmH\(_2\)O and \(V_T = 20/\text{ZEEP}\) groups (Figure 3).

![Figure 3](image)

*Figure 3. Volume/surface (V/S) ratio. V/S of the two ventilatory groups are exhibited: Group 1: Tidal volume \((V_T) = 10\) ml/kg and positive end-expiratory pressure \((\text{PEEP}) = 3\) cmH\(_2\)O \((V_T/\text{PEEP}).\) Group 2: \(V_T = 20\) ml/kg and \(\text{PEEP} = 0\) cmH\(_2\)O \((V_T/\text{zero end-expiratory (ZEEP)}).\) The results of the peri-atelectasis region (PeriAT) versus the contralateral lung region (C) are exhibited. Data are presented as medians and ranges. *\(P < 0.05.\)*

We did not find statistically significant differences between ROIs in tissue IL-1\(\beta\) or cytokine-induced neutrophil chemoattractant (CINC-1).

**Paper II**

At baseline measurements, no significant differences between groups were found in body weight, hemodynamics, and gas exchange. After establishment of the 2-hit injury VILI model, marked deterioration was seen: static
compliance decreased (28 ± 4.3 to 15 ± 1.8 ml/cmH2O) and the PaO2 /F1O2 ratio decreased from 63.2 ± 5.1 to 36.7 ± 13.2 kPa.

Extravascular lung water (EVLW) evolved differently over time in both groups (Figure 4)

![Graph showing EVLW measurements](image)

**Figure 4.** Evolution of the extravascular lung water (EVLW) measurements in both groups. Black circles correspond to the low respiratory rate group (LRR, 20 breaths/min), and white squares correspond to the high respiratory rate group (HRR, 40 breaths/min). Symbol * refers to P < 0.05 (Bonferroni’s post hoc analysis). Also, there is interaction between LRR and HRR group P < 0.01 (2-way ANOVA).

Pooling together all regional samples of each animal and then comparing HRR vs. LRR, the W/D ratio in the HRR group was greater than LRR (P < 0.001; Fig. 4). In the CV region, LRR presented higher W/D ratios than the rest of samples in the same group (P < 0.05), and levels similar to those in the HRR group (Figure 5)

![Box plot showing W/D ratios](image)

**Figure 5.** Comparison of wet-to-dry (W/D) weight ratio between both respiratory rate settings at the end of the experiment. LRR corresponds to the low respiratory rate group (20 breaths/min), and HRR corresponds to the high respiratory rate group (40 breaths/min). Symbol * refers to P < 0.05.
There was a higher peribronchiolar and perivascular leukocyte infiltration in the ventral region samples from the LRR group than in samples from the same region from the HRR group ($P < 0.05$) (Figure 6)

*Figure 6. Lung histological preparations. Panel A shows a section from caudal ventral (CV) region (10X) of a representative animal in the high respiratory rate group. Pulmonary edema appears as an amorphous, feathery eosinophilic material, barely visible, in the alveoli and the interlobular septum (black arrow). Note also a dilated lymphatic capillary in the septum (§) and low numbers of inflammatory cells in the alveoli and the septum. Two contracted bronchioles are visible (*). Panel B shows a section from CV region (10X) of a representative animal in the low respiratory rate group. Severe acute inflammation: alveoli display numerous leukocytes, predominantly polymorphs and macrophages, and also amorphous proteinaceous contents, consistent with a serous inflammatory exudate. A bronchiovascular unit displaying a collapsed bronchiolus is visible (*).

IL-10, IL-8, and TNFα concentrations in lung homogenates increased in response to LRR ($P < 0.05$, 2-way repeated-measures ANOVA). IL-6 showed interaction between RR and lung region ($P < 0.05$). BAL analysis showed higher concentrations of IL-1β in LRR ($P < 0.01$). Quantification of plasma cytokines demonstrated very low values that were under the detection limit in most samples, but did not show any difference between groups.

Immunohistochemistry of lung tissue exhibited higher pSmad2-stained cells in the HRR group compared with the LRR group ($P < 0.05$; Figure 7).
Figure 7. Representative lung sections from superior lobe (SA) from (A) LRR, low respiratory rate groups, and (B) HRR, high respiratory rate groups. Determination of pSmad2 as determinants of TGF-β pathway activation was done with the use of immunostaining. In blue: cellular nuclei (Hoechst 33342), and in red pSmad2 (+) nuclei (Alexa Fluor 568). pSmad2: phosphorylated mothers against decapentaplegic-2/3.

Paper III

No differences between the groups were found in body weight, hemodynamic parameters, and ventilatory measurements at baseline.

After the ARDS model was established, Cdyn,rs and oxygenation decreased markedly. But after the LRM (lung recruitment maneuver), both of them improved until they reached baseline values. Similar behavior was demonstrated by venous admixture and PaCO₂, and was similar in the two groups at all time points.

During the OLA protocol, heart rate increased in both groups over time. LRR, H0: 102 (24) to H6: 134 (25) bpm, and HRR H0: 111 (23) to H6: 143 (P < 0.0001). Systemic and pulmonary artery pressures decreased in both groups over time (P < 0.0001), without differences between groups. Cardiac output was not different between groups.

Extravascular lung water (EVLW) increased after inducing lung injury, but thereafter decreased progressively throughout the study period, with no differences between groups (Figure 8). Regarding W/D ratios, there was no difference between groups when all regional samples from each animal were pooled together, nor was there any difference when the different lung regions were analyzed separately.
Figure 8. A) Evolution of extravascular lung water (EVLW) in both groups. Black circles correspond to the low respiratory rate group (LRR, 20 breaths per minute) and white squares correspond to the high respiratory rate group (HRR, 40 breaths per minute). B) Comparison of wet-to-dry (W/D) weight ratio between groups at the end of the experiment. LRR corresponds to low respiratory rate group (20 breaths per minute) and HRR corresponds to high respiratory rate group (40 breaths per minute). Each point corresponds to one animal.

In addition, studied cytokines and histological analysis were not different between groups.

Paper IV

Baseline characteristics between both groups were not different regarding weight, hemodynamics and ventilatory variables. Induction of ARDS resulted in a marked decrease of respiratory system compliance and oxygenation.

After manual registration of a mean of 47 (43–67) landmarks in five animals from each group, we recognized an error in our method’s coregistration of 1.1 mm (0.57–2.21), but the error was the same in all groups.

Control animals presented a mean global deformation of 21 ± 11%, and ARDS animals presented a mean global deformation of 36 ± 23%. The strain maps showed heterogeneously distributed lung parenchyma deformations with a greater concentration of deformation in the gravitational intermediate part of the lung in both groups (P <0.05 1-way ANOVA) (Figures 9,10).
As previously reported, [60, 71] the global metabolic activity of the injured lungs was markedly increased, especially in the intermediate gravitational regions, as shown by the higher Ki values in comparison with controls.

We found a positive correlation between regional deformation and regional inflammation $R^2 = 0.58$ (0.02–0.84) for ARDS animals. This correlation improved to $R^2 = 0.85$ (0.47–0.97) when we excluded the three most dorsal ROIs from the analysis. In contrast, when Ki was correlated with the regional delta gas volume/regional end-expiratory gas volume ratio, we only found a weak correlation $R^2 = 0.33$ (0.04–0.72). Pearson's correlations between Ki and strain maps for individual injured animals are presented in Table 2. Analyzing control animals, individual correlations between lung deformation and inflammation were not evident $R^2 = 0.16$ (0.00–0.63) ($P > 0.05$).
Figure 10. Representative strain maps and $^{18}$F-FDG uptake (Ki) maps from the apical (upper) and basal (lower) lung region from: ARDS animal (left), and control animal (right).

Table 2. Pearson's correlations between Ki and strain maps (for the 10 ROIs), regional delta inspiratory gas volume/regional end-expiratory gas volume and strain maps excluding 3/10 dorsal ROIs. P1 to P7 represent each individual animal. (*) Corresponds to $P < 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Strain maps</th>
<th>Regional delta inspiratory gas volume/regional end-expiratory gas volume</th>
<th>Strain Maps 7/10 ventral ROIs</th>
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<tr>
<td></td>
<td>R$^2$ Ki/Deformation</td>
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<tr>
<td>P1</td>
<td>0.09</td>
<td>0.04</td>
<td>0.63*</td>
</tr>
<tr>
<td>P2</td>
<td>0.85*</td>
<td>0.72*</td>
<td>0.92*</td>
</tr>
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<td>0.51*</td>
<td>0.54*</td>
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<td>0.76*</td>
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<td>P5</td>
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<td>P6</td>
<td>0.84*</td>
<td>0.28</td>
<td>0.97*</td>
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<tr>
<td>P7</td>
<td>0.58*</td>
<td>0.33*</td>
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<tr>
<td>Median</td>
<td>0.58</td>
<td>0.33</td>
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Discussion

In the following discussion I number the studies according to the papers I-IV.

In the study reported in paper I, we used a novel model of non-lobar atelectasis induced by non-selective bronchial blocking in rats, and investigated its effects on the atelectatic area as well as on the surrounding healthy lung tissue during injurious mechanical ventilation. The atelectasis model was reliable, and greater histological evidence of hyperinflation and inflammation was observed in the PeriAT region. Most of the experimental studies on the relationships between atelectasis, mechanical ventilation, and VILI use animal models in which the lung injury is produced by surfactant depletion and/or some type of acute lung-insult[72]. For instance, surfactant depletion is usually produced by saline lavage and results in a decrease in lung compliance and hypoxemia[73]. When saline lavage is followed by mechanical ventilation with high volumes and low PEEP, a type of lung injury results that is very similar to ARDS in humans[72]. These models invariably contain, from the very beginning, unstable airspaces and consequently a gravitational gradient of collapse. In contrast, our experimental model was designed in such a way that the underlying process was, as far as possible, only the primary interaction between collapsed and aerated regions, that is, before any surfactant dysfunction and/or another potential VILI mechanism could become involved. Accordingly, our model initially presented lung parenchyma homogeneously aerated with an area of isolated peripheral inhomogeneity. In this way we could focus on the true interaction between collapse and healthy alveoli during the mechanical ventilation. VILI has been thought to predominate in the collapsed dependent regions and/or in the over-distended nondependent regions. Tsuchida et al. demonstrated that the atelectasis regions per se were protected from alveolar damage in a model of alveolar collapse and surfactant depletion, whereas alveoli from the aerated regions were more affected[74]. Very recently, Cereda et al. used magnetic resonance imaging to provide in-vivo imaging evidence of airspace expansion in a surfactant-depleted model[75]. Their data suggest that atelectasis could contribute to VILI by reciprocal increases in airspace size and that lung injury could be minimized through alveolar recruitment and adequate PEEP. This suggestion is supported by morphometric [74] and clinical data [76] and contrasts with previous reports suggesting an increase of VILI in atelectatic areas during MV[77, 78]. Adding another point of view regarding
the pressing question on the localization of the regional onset of lung inflammation, other recent findings suggest that the interface between the collapsed and aerated tissue may play an important role in lung injury[56]. Our findings, together with those of other investigators[29], suggest that the increased susceptibility to VILI is related to small-length-scale heterogeneities in the lung parenchyma. Indeed, Rausch et al., employing synchrotron-based X-ray tomographic microscopy on isolated rat lungs, estimated that local strains developing in alveolar walls are as much as 4 times higher than the global ones[34]. Their data suggest that thin regions may become over-stretched, whereas regions with tissue accumulation remain unchallenged. These data fit with our own results and strongly suggest that a tidal stretch of the healthy aerated parts can play a primary role in the activation of the inflammatory signaling cascade[27, 60, 76]. It seems likely that the collapsed region may indirectly damage the surrounding initially healthy aerated tissue, acting as a stress riser. We found a concentration of mechanical trauma (alveolar wall disruptions and signs of over-distension) and inflammation (neutrophilic infiltration and interstitial edema) in the region surrounding the atelectasis. In their theoretical analysis, Mead et al. estimated that the alveolar pressure at the junction of the fully collapsed and expanded alveoli could be as high as 4 to 5 times the applied pressure[33]. This landmark estimation of Mead of approximately 4 times local amplification was recently confirmed using synchrotronbased X-ray tomographic microscopy in a preparation of rat lungs[34], as discussed above. Similarly, Rouby et al. described that in an autopsy study[79] expanded pseudocysts were concentrated around atelectatic lung regions. We found evidence of inflammation in the atelectasis region (intravascular neutrophils and tissue cytokines); however, we did not identify signs of alveolar disruptions in this area. We speculate that the inflammation could be due to vascular injury secondary to high blood flow in a systemic inflammatory scenario that had induced endothelial activation and neutrophil adhesion, explaining the inflammatory signs in this non-ventilated portion of the lung. We did not find significant differences in cytokine concentrations between PeriAT and control regions in the two ventilatory groups. We can postulate two possible explanations: A) the duration of the protocol was too short, and B) the technique of tissue sampling may have been unable to accurately represent the two ROIs. This study has some limitations. First, the region of atelectasis was induced exclusively in the basal region of the lung. However, the anterior or posterior location could not be selected due to the methodology and setup we used. Bronchoscopy-guided plugs applied in larger animals may be a suitable alternative, allowing more topographical options for the induction of atelectasis and better reproduction of clinically relevant atelectasis in healthy lungs. Second, the duration of the ventilatory protocol was 180 minutes. A longer protocol would perhaps have allowed more precise study of the inflammatory response patterns and the relationships between the ROIs. Third, a non-lobar atelectasis model was
used that is different from the dependent collapse characteristically observed in ARDS patients. It was, however, worthwhile to assess the spatial and time effects of localized inhomogeneity from their beginning without the concomitant presence of other sources of airspace instability. Fourth, although silicon cylinder blockers are biocompatible and used in the clinical setting, we cannot ignore the fact that the blockers could induce some degree of airway and or parenchymal trauma. Finally, in an ex-vivo lung injury model, the presence of atelectasis was associated with higher transcription of stress kinases and histological evidence of lung damage in the context of protective mechanical ventilation (low tidal volume)[80]. This finding suggests that a control for future work could be a condition characterized by the presence of atelectasis without mechanical stress (for instance, using a continuous positive airway pressure mode).

Thus, in the study reported in paper I we found that local non-lobar atelectasis could act as a stress concentrator, generating structural alveolar injury and inflammation in the surrounding lung tissue.

In the study reported in paper II evaluated the isolated and independent effect of two clinically relevant respiratory rates on the evolution of early experimental ARDS during the protective ARDSNet strategy. HRR was associated with increased pulmonary edema and higher activation of the TGF-β pathway, whereas LRR showed evidence of higher inflammation.

This experimental model, saline lavage followed by injurious mechanical ventilation with high volumes and low PEEP, was developed to reproduce as faithfully as possible the full characteristics of early human mild/moderate ARDS. Surfactant depletion by saline lavage results in a decrease in lung compliance and hypoxemia[73] but not in alveolar epithelial injury or neutrophilic alveolitis[81]. When saline lavage is followed by injurious mechanical ventilation, it results in a type of lung injury that is very similar to ARDS in humans[72]. Thus, the model we used results in heterogeneously aerated lungs with significant amounts of non-aerated lung tissue, tidal R/D, hyperinflated lung tissue, tidal hyperinflation, and regionally distributed lung inflammation[60, 71]. In accordance with previous studies, we found that lung edema and inflammation develop within 6 hours after establishing a lung injury model[60, 71, 82-84].

To date, the effect of RR has not been studied in clinical trials. In protective ventilation strategies trials RR have been assigned only on the basis of pH levels, and/or the presence or not of intrinsic PEEP, and/or an arbitrary upper limit for RR. Importantly, none of these criteria can discriminate any independent and specific effect of RR on VILI.

Our data showed a higher concentration of some cytokines in lung parenchyma from the LRR group. However, the concentrations of cytokines from most plasma samples were under the detection threshold, suggesting that the inflammation was minor and/or very compartmentalized. Moreover, the cy-
tokine levels reflect only the early phase of ARDS, and not the whole evolution of ARDS.

The higher degree of inflammation in the LRR group compared with HRR group could be explained by two possible mechanisms: 1) by possible cyclic recruitment/decruitment (R/D) and the somewhat higher driving pressures inducing inflammation in the LRR group[85, 86], or 2) TGF-β "context-dependent" actions and/or anti-inflammatory effects induced by the higher TGF-β concentration downregulating inflammation in the HRR group. The mechanism is context dependent because TGF-β can either inhibit or enhance effector function over inflammatory cells depending on the environmental cytokines profile. High concentrations of TGF-β, as were observed in the HRR group, can suppress and antagonize production of several cytokines from macrophages and NK cells. The importance of TGF-β in limiting inflammation is clearly demonstrated in TGF-β-knockout mice, which die at 3–4 weeks of age from a wasting syndrome associated with multifocal inflammatory disease[87].

In contrast to this study, several experimental studies have suggested that a low respiratory rate reduces inflammation, edema, and alveolar damage[40, 42, 45, 46, 88]. However, most of these studies were performed in rodents and, furthermore, did not fully control tidal volumes, inspiratory times and, importantly, inspiratory flows, which were lower in the LRR group in our study. Indeed, limiting inspiratory flow has been shown to be highly lung protective when a volume-controlled pressure-limited mode is used[46]. In addition, most of these studies examined extreme values of RR[42, 44-46]. In our study, the only difference between the groups was a clinically relevant RR. However, since both groups had identical inspiratory flow, inspiratory time, PEEP, and tidal volumes, minute ventilation was doubled in the HRR group compared with the LRR group. As could be expected, the low minute ventilation associated with the LRR strategy resulted in hypercapnic acidosis. Hypercapnia can reduce inflammation and lung injury as has been shown in several experimental models[89, 90]. Therefore, and in order to keep PaCO₂ and alveolar ventilation similar in both groups, we added an extra instrumental dead space in the HRR group, eliminating the confounding effect of hypercapnic acidosis. Another option would have been to add exogenous CO₂ to the inspired gas, instead of increasing the flow of rebreathed CO₂ to the HRR group. This would have eliminated the need of an instrumental dead space, but would not otherwise have influenced the study design or results.

Hyperventilation has been shown to have deleterious effects, not only during mechanical ventilation, but also in spontaneously strenuously breathing healthy animals[91]. Moreover, ultraprotective mechanical ventilation strategies suggest that "less ventilation, less injury"[92-94]. However, in these experiments, it was not possible to determine whether the initiating
cause of VILI development was the effect of VT, RR, minute ventilation, or hypocapnia.

Baumgardner et al. have shown that the magnitude of PaO\(_2\) oscillations decreases with increasing RR[95], suggesting that the dynamics of the lungs (i.e., RR and expiratory time) and not just the statics of the lungs (i.e., the PEEP levels) are important for cyclic recruitment/derecruitment. Lung dynamics is a function of regional alveolar tissue mechanics, regional compliance, regional airway resistance, and gas/liquid interfacial mechanics that determine the time constants for regional lung collapse. Thus, a high RR reduces the available time for expiratory collapse and, thus, prevents cyclic R/D at low extrinsic PEEP[95]. In addition, a possible explanation is that in the HRR group intrinsic PEEP occurred in lung regions with longer time constants, thereby reducing regional derecruitment. In fact, Neumann et al. showed by dynamic computed tomography scanning in oleic-acid injury in pigs that time dependent collapse was prevented by reducing the expiratory time[96]. Recently, the same group found in a surfactant-depleted model that the reverse was true, i.e., that prolonged expiratory time promoted alveolar collapse, cyclic R/D, and also the redistribution of regional ventilation to non-dependent regions[97]. In our study, the expiratory time was almost doubled in the LRR group, and it is conceivable that this caused a higher degree of collapse, which is also indicated by the lower respiratory compliance. This implies that the end-expiratory lung volume was lower in the LRR group, and therefore the \(V_T\) was distributed in a smaller lung volume, thereby generating higher dynamic strain[28] and thus injury in the ventilated lung regions. In fact, in the LRR group, inflammation was greatest in the ventral, non-dependent, i.e., ventilated lung regions, and not to the collapsed, i.e., not ventilated, lung regions[97]. Notwithstanding, another possible mechanism for more inflammation in the low LRR group is a difference in driving pressures (\(\Delta P\)). \(\Delta P\) is the difference between end-inspiratory and end-expiratory pressure and may be an especially interesting clinical estimate of lung stress as it is inversely proportional to respiratory system compliance. When analyzed during constant flow ventilation, \(\Delta P\) contains implicit information regarding how changes in PEEP modulate cyclic stretch. Tidal volume/dynamic strain, when normalized for lung compliance (i.e., \(\Delta P\)), contains additional useful information regarding the interplay between \(V_T\), plateau pressure, and PEEP and their role in VILI[28, 98]. In our study, the LRR was associated with significantly higher \(\Delta P\). Amato et al. examined \(\Delta P\) as an independent variable associated with survival in ARDS patients. Their analyses indicated that reductions in \(V_T\) or increases in PEEP were beneficial only if associated with drops in \(\Delta P\), and that no other ventilation variable exhibited such a mediating effect[85].

The HRR lungs presented higher water content than the LRR at the end of experiment. Observing the temporal changes of EVLW, we interpret these findings not just as a gaining of water but also as a decrease in the ability to
clear lung edema. The primary mechanism responsible for the resolution of alveolar edema is active vectorial sodium transport across the alveolar epithelium[99]. In ARDS, factors that may impair the resolution of alveolar edema are hypoxia, high tidal volume, and several cytokines such as IL-1β, IL-8, and TGF-β. TGF-β is a cytokine implicated in inflammation, fibrosis, and wound repair processes, and it is activated by, e.g., mechanical strain. TGF-β signaling proteins pSmad2/3 promptly reduce trans-epithelial sodium transport by inducing endocytosis of the αβγ epithelial sodium channel complex (ENaC) and then promoting alveolar flooding and persistence of pulmonary edema. Recently, Peters et al. demonstrated that BAL fluids from ARDS patients rapidly led to ENaC internalization on lung epithelial cell cultures[65]. This pathway drives a rapid (within 30 min) and dramatic (>80%) reduction in the cell-surface abundance of ENaC. Administration of TGF-β to rabbits and rats blocked the sodium transport and caused fluid retention[65, 100]. Corroborating the role of TGF-β in ARDS, two studies demonstrated increased TGF-β levels in lung fluids from patients with ARDS, and, furthermore, lower TGF-β levels correlated with more ventilator-free and intensive care unit-free days[101, 102]. We assessed the activation of this pathway in both RR groups. By immunohistochemical analysis, a higher activation in the HRR group was evident. The continuous higher degree of mechanical strain in the HRR group may be the reason for this finding and may suggest a possible mechanism for the impairment of lung water clearance associated with HRR.

The HRR group presented higher respiratory system compliance and consequently larger lung volume. Egan et al. studied alveolar permeability at various levels of lung inflation in adult sheep[103]. Their results demonstrate that the epithelium becomes permeable to larger molecules as inflation volume is increased. Moreover, the inflation changes in epithelial permeability appeared restricted to situations in which only a fraction of the lung expands in response to high pressures[103-105], such as in the clinical situation of respiratory failure as well as in our experimental study, where much of the air space is collapsed. Along the same lines, Bhattacharya et al. suggested that high lung inflation contracts the interstitial liquid channels in edematous lungs and, consequently, decreases the ability to clear edema[106]. Therefore, the higher water content of our HRR lungs may also be related to a larger, more inflated, lung volume.

In conclusion, high respiratory rate was associated with more pulmonary edema and higher activation of the TGF-β pathway, whereas low respiratory rate was associated with higher lung inflammation.

In the study reported in paper III, we observed no influence of respiratory rate on lung inflammation or edema when an OLA strategy was used[107], in contrast to Study II in which we applied an ARDSNet strategy.

Protective mechanical ventilation strategies may have some side effects that can be potentially harmful to the lungs. The common lung protective
ventilation strategies, such as those proposed by the ARDSNet, limit volume and pressure, but apply only moderate levels of PEEP according to oxygenation. Therefore, these approaches do not avoid alveolar collapse, heterogeneity, or unstable alveoli[55]. Higher regional lung strain and heterogeneity could cause overdistension, barotrauma, and inflammation in already injured lung tissue as well as in healthier lung regions[34, 108]. Furthermore, to maintain sufficient levels of ventilation, such strategies often require increasing the respiratory rate. The respiratory rate may constitute an amplification factor of the cumulative energy absorbed by the lungs with each respiratory cycle, and theoretically, in the described scenario of heterogeneous and unstable lung parenchyma, could be a pivotal factor in the development of VILI.

The OLA strategy aims to recruit previously closed alveolar units by transiently applying high alveolar pressures, and thereafter, keeping them open by applying PEEP levels high enough to avoid collapse. This requires higher pressures than those usually applied during conventional PEEP settings[31, 84]. By maximally recruiting lung tissue, the OLA reduces opening and closing phenomena and decreases lung parenchyma heterogeneity[58]. In this way, it may theoretically decrease the amount of energy absorbed by each lung unit in each respiratory cycle.

In this study, we applied an OLA strategy consisting of a LRM followed by a rather high PEEP set according to best compliance. The LRM used sought to reopen most of the collapsed lung parenchyma; we confirmed maximal lung recruitment by analysis of arterial blood gases. We considered full lung recruitment when the sum of pCO₂ and pO₂ was above 53 kPa[57]. In addition, we performed a pilot experiment in which we performed repeated CT scans in our ARDS model, confirming significant recruitment and homogenization of the lung parenchyma after applying this OLA strategy. The full lung recruitment obtained with our OLA disagrees with the finding in a recent study by Grasso and coworkers, who showed that an open lung approach was not able to homogenize the lung parenchyma and attained only partial lung recruitment[31]. However, the methodology applied in Grasso et al.’s study differs in several aspects from ours because they used a LRM targeted at a transpulmonary pressure of 30 cmH₂O for 40 to 60 seconds, whereas we used an inspiratory pressure of 55 cmH₂O with a PEEP of 40 cmH₂O for 120 seconds. Moreover, the PaO₂/FiO₂ ratio was markedly higher at the end of our study, and we titrated PEEP to an optimal value according to the best compliance. This level has been shown to prevent end-expiratory collapse of the recruited lung[109]. This, in fact, allowed us to ventilate with a much lower driving pressure after applying the OLA (6 versus 15 cmH₂O) for the same applied tidal volume. We are therefore confident that our OLA strategy resulted in an efficient homogenization of the lung parenchyma and a high degree of alveolar stabilization. However, we did not assess cyclic R/D or overdistension by CT imaging or stress index[97].
The lack of differences between groups in Study III acquires more relevance when this finding is contrasted with those obtained in Study II, in which an ARDSNet strategy was used. Taking into account the limitation that we compared two separate experimental protocols, it is important to remark that both protocols were done with the same methodology, i.e., we used the same ARDS model, which resulted in comparable changes in respiratory mechanics and blood gases, and the respiratory rates explored were identical. The management of fluids and circulatory parameters was similar. The only difference was the strategy used to set PEEP and the use of LRM. We observed that when using an OLA strategy, RR did not influence EVLW evolution, and that both the HRR and LRR groups in the present study exhibited the same behavior as the group LRR-ARDSNet in the Study II. In contrast, the HRR-ARDSNet group showed a higher EVLW at the end of the study (Figure 11). Comparing W/D weight ratios, we observed a similar trend, with a higher value in the HRR-ARDSNet group. These results suggest an interaction between the recruitment strategy and the effect of the respiratory rate on lung edema.

![Figure 11. Relative changes in EVLW. Evolutionary effects of both respiratory rates (HRR = 40 bpm and LRR = 20 bpm) comparing OLA (this study) and ARDSNet strategies](image)

Egan suggested that edema formation was related to the level of lung inflation in relation to lung compliance,[60, 103-105, 110]. In other words, in injured and collapsed lungs, $V_T$ will induce a high level of inflation in the functional residual lung, increasing its permeability. However, if the collapsed lung is recruited and kept open according to the best $Crs$, the same $V_T$ will induce proportionally less tissue deformation, and consequently, less edema.
In Study III, we did not find differences in the lung inflammatory response between the HRR and LRR groups. In Study II using the ARDSNet strategy, we interpreted the presence of more inflammation in the LRR group as a consequence of an increased likelihood of end-expiratory collapse and hence of the occurrence of cyclic opening and closing of alveoli. This principle was recently well documented by Boehme et al. in a model of surfactant depletion, in which they observed higher \( \text{pO}_2 \) oscillations related to cyclic opening and closing using longer expiratory times[97]. In Study III, the higher PEEP levels used, adjusted to prevent the end-expiratory collapse of the lung, may well have prevented end-expiratory collapse in the LRR group, despite the longer expiratory times. Moreover, in Study II, cytokine levels in the non-dependent collapsed regions were up to 100 times higher than in the dependent or intermediate regions. This is in contrast to the study III, in which tissue cytokine expression was homogeneously distributed along the three gravitational regions assessed. These findings support the concept of lung homogenization attained by the OLA strategy and thus a more homogeneous distribution of stress over the lung parenchyma. Maximal lung recruitment and careful PEEP selection, such as performed in Study III, promote an active modification of the lung’s mechanical scenario, redistributing the \( V_T \) from overdistended to newly opened lung regions[57]. The gain in lung compliance after a successful recruitment rescales, i.e., increases the size of the functional lung, potentially allowing for a reduction in driving pressures without decreasing ventilation.

Recently, Amato and coworkers provided evidence that driving pressure, as a determinant of dynamic strain[28], was the main determinant of outcome in patients with ARDS[85]. In Study II, the resulting driving pressures were almost double those obtained in Study III. The very low driving pressures generated in both groups in the study reported in paper II, using OLA may indicate that dynamic strain was equally low in both groups and may explain why respiratory rate had no deleterious effect on lung tissue. This notion agrees with a recent study by Cressoni et al., who found in a pig model that ventilation with a power (the product of \( V_T \), driving pressure and RR) below a specific threshold does not induce VILI[111].

In conclusion, in Study III we found that the respiratory rate had no influence on the lung inflammatory response, edema, or fluid resorption when an OLA strategy was used in an animal model of ARDS. This indicates that OLA ventilation, by keeping the lungs open, decreases dynamic stress, thereby reducing the effect of respiratory rate on the development of VILI.

Studies II and III share similar limitations. First, the duration of the experimental protocol was short and allowed evaluation of only the beginning of lung edema formation/reabsorption and the early inflammatory process. Thus, our results in Studies II and III mainly reflect the very early changes associated with the use of two respiratory rates, and we do not know whether these results would remain the same during a longer protocol. Second, our
ARDS model is an animal model with all the inherent limitations of such a model, and although the model mirrors many of the characteristics of ARDS, it is not possible to translate our findings directly to patients. Importantly, this model reflects homogenously injured lungs such as seen in early ARDS, with high recruitability, and may not be valid for other ARDS conditions. Third, due to the limited number of animals, we cannot exclude the presence of minor differences between groups with regard to inflammation and edema. Fourth, although we kept PaCO2 levels similar by adding external dead space, this does not exclude differences in regional alveolar CO2 tensions between groups. Fifth, we measured lung water but not clearance or filtration, so the mechanism of lung edema cannot be definitely explained.

In the study reported in paper IV showed a positive spatial correlation between regional volumetric strain and inflammation in a porcine ARDS model. Regional volumetric strain was assessed from CT images by using a novel mathematical approach, showing a heterogeneous distribution of lung parenchyma deformation, with a greater concentration in the gravitational intermediate zone of the lungs.

The experimental model of lung injury used in Study IV is more severe than the one used in Studies II and III. It has some relevant characteristics that are important to note: 1) it reproduces several characteristics of ARDS as heterogeneously aerated lungs with significant amounts of non-aerated lung tissue, tidal R/D, hyperinflated lung tissue, and tidal hyperinflation; 2) it presents heterogeneous distribution of lung inflammation [84, 107]; 3) it is characterized by significant alterations in gas exchange, mechanics, and hemodynamics, with a clear differentiation compared to healthy controls [60, 71, 84, 107].

Computation of pulmonary strain is a complex process, and in an attempt to simplify it, strain has been described as lung deformation or “volumetric” strain, the ratio of volume of gas inflated to functional residual capacity (VT/FRC) [26, 28]. This approach makes it possible to recognize the global strain's threshold (whole lung) where development of VILI starts [112]. Also, this kind of analysis has been applied in the regional study of ARDS lungs, but its results are difficult to interpret, especially in the dorsal part of the lungs, because the tidal reopening of collapsed dorsal lung regions during inspiration could push the VT/FRC ratio value to infinite [113].

Over the past few years, with the advent of advanced CT and image processing techniques, several new methodologies have been developed to obtain the displacement of voxels from two lung images at different levels of ventilation in a non-invasive fashion [70, 114, 115]. More recently, 3-D maps of regional volumetric strain have been obtained from high-resolution CT images that have confirmed the large heterogeneity of volumetric strain in healthy human lungs with high fidelity [116, 117]. Briefly, our new methodology uses an automatic recognition and co-registration of numerous set points (voxels patterns) between the inspiratory and expiratory CT images;
this process proved to be very accurate when we compared it to our manually-defined landmarks [118]. It also showed better performance and less registration error when compared with the previously available methodologies such as B-spline interpolations [69].

There is a physiological link between lung deformation and lung inflammation. At a cellular scale, the exaggerated elongation of cell membranes, cytoskeleton, and organelles may result in disruption of these structures and exposure of intracellular structures and molecular patterns (DAMPs). This will activate the innate immune system and recruit neutrophils, which are known to play an important role in the early phases of lung inflammation during ARDS [72, 119-121]. Dynamic PET with $^{18}$F-FDG can be used to image cellular metabolism, which, during lung inflammatory processes, mainly reflects neutrophil activity [119]. Previous studies in which dynamic PET with $^{18}$F-FDG was used in this same experimental model have shown that inflammation was higher within the gravitationally intermediate zones, where the normally and poorly aerated regions predominate [60].

There have been several efforts to correlate topographically potential mechanisms of VILI with regional lung inflammation. Bellani and coworkers studied regional R/D through identification of regional changes of non-aerated parenchyma, and regional inflammation using 18-F-FDG, but they did not find any correlation [76]. More recently, in an experimental model of lung injury, Wellman and coworkers assessed lung strain, expressed as sVol, (defined as the change in gas volume during inspiration divided by end-expiratory gas volume) in six ROIs, as determined by three gravitational levels, and a cephalic/caudal division. They used models of lung injury based on injurious ventilation with or without an intravenous infusion of LPS. They reported a linear correlation between regional strain and inflammation ($^{18}$F-FDG Ki). Additionally, they reported that gravity had an influence on sVol and Ki, with the highest values of sVol and Ki in the dependent regions [29]. Their findings are consistent with our data; however, we observed the highest deformation in the intermediate lung regions, while they observed it in the dorsal lung regions. This could be explained by differences in the experimental models. The surfactant depletion used in our study induces important dorsal collapse, decreasing lung compliance in this part of the lungs [122].

We found greater deformation and more inflammation in the gravitationally intermediate lung regions, and a good correlation between them. However, the correlation between deformation and inflammation was lower when the dorsal lung region was included in the analysis. Frequently in this surfactant-depletion model, dependent lung regions are collapsed during the expiratory phase, but high tidal volumes, such as those we used, induce marked changes on the distribution of CT densities within those regions,[123] worsening the quality of the co-registration process.
Some limitations of the PET technique we used have been discussed in detail previously [60, 66, 71, 124, 125]. In particular, our study has various limitations that are worthy of note: 1) We used images obtained from a previous study. 2) Animal models of ARDS do not fully reproduce the findings of human disease [72]. 3) We did not study the distribution of perfusion in the lung, which is usually not homogeneous [126, 127], and might be associated with the distribution of $^{18}$F-FDG uptake within the lung [128]. However, this is more likely to represent an epiphrenomenon in conditions of endotoxemia than a bias in the measurement of the $^{18}$F-FDG distributions, because in other circumstances $^{18}$F-FDG uptake has been repeatedly shown to be unrelated to regional perfusion [129-131]. Importantly, in experimental models of unilateral lung injury [66, 125], the injured lung always showed greater $K_i$ than the control lung, independent of whether blood flow favored the injured lung or not. 4) We did not obtain PET/CT data at baseline or immediately after inducing ARDS. The $^{18}$F-FDG PET scans were acquired only after injury because of feasibility reasons (the 110 min half-life of $^{18}$F-FDG precluded more injections of the isotope during the study time available). 5) The method for biomechanical analysis of CT lung images suffers from some limitations, which I briefly describe next. The accuracy of the finite-element method used to compute the volumetric strain map strongly depends on the quality of the image registration method. Errors in the registration process can only be identified in a semi-automatic way, by visually comparing the resampled image with the target image. While we have checked for the registration quality using such an approach and have performed a second image registration with different parameters in the case of blurry resampled images, it is impossible to ensure that the registration error will be at the same level for all images analyzed. Therefore, although the finite-element biomechanical analysis employed in this work has been shown to be more accurate than traditional methods based on direct differentiation [69], we expect some intersubject variability in the error of the biomechanical analysis.

In conclusion, Study IV suggests that local lung deformations correlate well with local inflammation in ARDS. The largest stretch was concentrated in the intermediate gravitational zones of injured heterogeneous lungs.
Conclusions

My thesis in experimental animal models showed the following:

I. Local non-lobar atelectasis can act as a stress concentrator, generating structural alveolar injury and inflammation in the surrounding lung tissue.

II. When the ARDSNet ventilatory strategy is used in the acute respiratory distress syndrome, a high respiratory rate is associated with more pulmonary edema and higher activation of the TGF-β pathway, whereas low respiratory rate is associated with higher lung inflammation.

III. When an open lung approach ventilatory strategy is used in the acute respiratory distress syndrome, the respiratory rate does not influence the lung inflammatory response, edema, or fluid resorption. This indicates that open lung approach ventilation, by keeping the lungs open, decreases dynamic stress, thereby reduced the effect of respiratory rate on the development of ventilator-induced lung injury.

IV. Local lung deformations correlate well with local inflammation in the acute respiratory distress syndrome. The largest stretch is concentrated in the intermediate gravitational zones of injured heterogeneous lungs. This new image-based estimation of regional volumetric strain, based on finite element interpolations, has the potential to give new insights into local pathogenic mechanisms of ventilator-induced lung injury, and to become a tool to assess protective ventilatory strategies.
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)