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Protective Mechanical Ventilation in Inflammatory and Ventilator-Associated Pneumonia Models

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Abstract

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Severe infections, trauma or major surgery can each cause a state of systemic inflammation. These causes for systemic inflammation often coexist and complicate each other. Mechanical ventilation is commonly used during major surgical procedures and when respiratory functions are failing in the intensive care setting. Although necessary, the use of mechanical ventilation can cause injury to the lungs and other organs especially under states of systemic inflammation. Moreover, a course of mechanical ventilator therapy can be complicated by ventilator-associated pneumonia, a factor greatly influencing mortality. The efforts to avoid additional ventilator-induced injury to patients are embodied in the expression 'protective ventilation'.

With the use of pig models we have examined the impact of protective ventilation on systemic inflammation, on organ-specific inflammation and on bacterial growth during pneumonia. Additionally, with a 30-hour ventilator-associated pneumonia model we examined the influence of mechanical ventilation and systemic inflammation on bacterial growth. Systemic inflammation was initiated with surgery and enhanced with endotoxin. The bacterium used was *Pseudomonas aeruginosa*.

We found that protective ventilation during systemic inflammation attenuated the systemic inflammatory cytokine responses and reduced secondary organ damage. Moreover, the attenuated inflammatory responses were seen on the organ specific level, most clearly as reduced counts of inflammatory cytokines from the liver. Protective ventilation entailed lower bacterial counts in lung tissue after 6 hours of pneumonia. Mechanical ventilation for 24 h, before a bacterial challenge into the lungs, increased bacterial counts in lung tissue after 6 h. The addition of systemic inflammation by endotoxin during 24 h increased the bacterial counts even more. For comparison, these experiments used control groups with clinically common ventilator settings.

Summarily, these results support the use of protective ventilation as a means to reduce systemic inflammation and organ injury, and to optimize bacterial clearance in states of systemic inflammation and pneumonia.

Keywords: Mechanical ventilation, Systemic inflammation, Pneumonia, Ventilator-associated pneumonia, Endotoxin

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To my family; Linda, Clint and Frank

List of Papers

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

I Sperber J, Lipcsey M, Larsson A, Larsson A, Sjölin J, Castegren M: **Lung Protective Ventilation Induces Immunotolerance and Nitric Oxide Metabolites in Porcine Experimental Postoperative Sepsis.** *PLoS ONE* 2013, 8:e83182.

II Sperber J, Lipcsey M, Larsson A, Larsson A, Sjölin J, Castegren M: **Evaluating the Effects of Protective Ventilation on Organ-Specific Cytokine Production in Porcine Experimental Postoperative Sepsis.** *BMC Pulm Med* 2015, 15.

III Sperber J, Nyberg A, Lipcsey M, Melhus Å, Larsson A, Sjölin J, Castegren M: **Protective Ventilation Reduces *Pseudomonas Aeruginosa* Growth and Lung Injury in a Porcine Pneumonia Model.** Submitted manuscript.

IV Sperber J, Nyberg A, Lipcsey M, Larsson A, Sjölin J, Castegren M: **Exposure to Mechanical Ventilation and Endotoxin for 24 Hours Before Infection Influences *Pseudomonas Aeruginosa* Growth During Experimental Porcine Ventilator-Associated Pneumonia.** Manuscript.

Contents

Abbreviations

ALI	Acute Lung Injury
AM	Alveolar macrophage
ARDS	Acute Respiratory Distress Syndrome
BAL	Bronchoalveolar lavage
CARS	Compensatory Anti-inflammatory Response Syndrome
cfu	Colony forming unit
CI	Cardiac index
ET	Endotoxin tolerance
FiO_2	Inspired oxygen fraction
FRC	Functional residual capacity
i.e.	Id est
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MAP	Mean arterial pressure
MPAP	Mean pulmonary arterial pressure
n	Number
NO	Nitric oxide
$PaCO_2$	Arterial tension of carbon dioxide
PAMP	Pathogen-associated molecular pattern
PaO_2	Arterial tension of oxygen
PCWP	Pulmonary capillary wedge pressure
PEEP	Positive end-expiratory pressure
PRR	Pattern recognition receptor
SIRS	Systemic Inflammatory Response Syndrome
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
VAP	Ventilator-associated pneumonia
V_T	Tidal volume

Introduction

This thesis is about breathing and inflammation. Or more truthfully, it is about artificial breathing and severe systemic inflammation. And about the mix of the two. And then about some bacteria too. Anyhow, these subjects are of paramount importance to humans.
Breathing is necessary for upholding life, and by implication artificial mechanical ventilation when a person cannot breathe was long considered as simply good. This was in spite of experimental evidence to the contrary. The insight that mechanical ventilation could contribute to the death of patients was fully embraced by the intensive care community only at the shift of the millennium. Since then, the practice of how to mechanically ventilate has substantially changed and the concept of **protective ventilation** with the aim to avoid causing injury has been an expanding research area.

One reason for the slow awakening could be the fact that it is hard to conduct research studies on intensive care patients, and results become the subject of debate. As a group patients are heterogeneous in regards to age, comorbidities, genetics, state of inflammation, physiologic reserves etc. In practice, this calls for very large numbers of patients to be included in studies to get intelligible results. Although animal experimental designs have shortcomings, they can avoid many of the factors of heterogeneity found in patients. Therefore, they can be used efficiently to examine basic physiological or immunological reactions and to evaluate different forms of interventions.

Inflammation is a basic host response to threats such as infections or mechanical or chemical injury. It is essential for survival and healing. Its role as a key player in severe illness has grown with the emergence and development of modern intensive care, and with the identification and description of inflammatory mediators. Today, patients are kept alive long enough by artificial means to develop multiple organ failure, ventilator-associated pneumonia, and secondary or tertiary sepsis. They tend to die from the after-effects of trauma or severe infections, and not from the initial insults. These complications and deaths are heavily influenced by disturbances in the immune system functions such as inflammation. Many efforts have been conducted to try to control the inflammatory response in critical illness. These have failed to produce a universal cure, likely due to the complex function of inflammation and the lack of clear definitions of different inflammatory

states. Likely, future treatments have to be more adapted to the individual and more flexible.

Too much inflammation and too little is bad for outcome. We have focused on reducing the initial 'too much' part in this thesis. The four experiments presented are animal models molded to be relevant to clinical scenarios in intensive care. These areas are post-operative sepsis, pneumonia, and ventilator-associated pneumonia. Our main objective has been to evaluate the impact of mechanical ventilation, especially protective ventilation, under these conditions. Our main hypotheses have been that protective ventilation will reduce systemic and organ-specific inflammation, reduce organ damage, and additionally reduce the bacterial burden in the lungs during pneumonia.

Animals in research

Background. Jeremy Bentham famously proposed in 1789, that animals should have rights based on their ability to suffer [1]. However, the belief that animals did not feel pain persisted through most of the 19th century. Before the discovery of anesthesia in 1846 all animal experiments were performed without pain-relieving measures. Legislation to protect animal rights in Victorian England was heavily influenced by the Royal Society for the Prevention of Cruelty to Animals. The Cruelty to Animals Act of 1876 posed restrictions on experiments involving extreme pain or unnecessary duplication during the practice of vivisection, important to those engaged in medical research. It has been proposed that the horrors induced by vivisection reflected the uncertainty of the role of science in society and concern over a potential loss of compassion among medical professionals [2]. At the time of the First World War, animal movements had lost momentum partly due to medical advances made possible by animal experiments, i.e. vaccines.

The United States followed behind England, forming the American Society for the Prevention of Cruelty to Animals in 1866, later to be incorporated into the umbrella organization of the American Humane Association. The rise of anti-vivisection organizations found hard opposition from the medical associations - the National Academy of Sciences and the American Medical Association. An organization with the purpose of securing the future of animal research was founded in 1908 – Council on Defense of Medical Research. The stage was set for continuing conflict. However, a period of stagnation in the progress of animal rights may have been due to two World Wars. The civil rights movement of the 1960's saw parallels between gender-race discrimination and ill treatment of animals – termed "speciesism" and popularized by Peter Singer [3]. Singer has been greatly influential in melding human biomedical ethics with concern for animal welfare, proposing consistency by minimizing suffering of sentient life. The Institute of Laboratory Animal Resources produced the first edition of the *Guide for the Care and Use of Laboratory Animals (Guide)* in 1963. It is regularly updated and used as a worldwide standard for laboratory animal care and use programs [4]. Two high profile media cases in the early 1980's were pivotal to enforce practical change. These changes included that Institutional Animal Care and Use Committees had to conduct prior reviews of all animal experiments to minimize pain and that ethical scales were introduced to guide the assessments. The infliction of pain was rarely justified, and then only by the

quality and importance of the scientific objective of the study. The new policies were implemented in the 1985 version of the *Guide*.

It has long been taught and adopted the view that Science is value free in general and ethics free in particular, and that it does not make moral judgments but deals with facts. Ethical considerations have therefore not been integral parts of the development of natural sciences since the renaissance. Rather, ethics has been regarded as a hindrance or a restraint upon science, and as such met with wild disapproval from the scientific community. A large influence on scientific thinking comes from the **Logical positivist** movement of the 20th century that proposed, in summary, that proper science should not allow unverifiable statements. Only that which can be proved or disproved empirically has any real meaning. Through adopting this view one could with good conscience dismiss claims of religious or metaphysical nature, such as animal ethics, as irrelevant to science, and to top it off - as meaningless [5]. Positivism is arguably still today the dominant scientific view in natural sciences, but biomedical and animal ethics are taught actively to the new generations of scientists and are considered integral parts of science.

Rules and regulations. The Swedish Board of Agriculture regulates the use of experimental animals. An experimental animal is bred for the purpose of experiments. They can be used for scientific research experiments, surgical training, and development of pharmaceuticals or research advancing animal welfare. The latter does not necessarily include any form of suffering from the animal [6]. Three R's formulated by the scientists Russel and Burch in 1959 form widely adapted guiding principles in research involving animals; **Replace** animal experiments with a different method when possible, **Reduce** the number of animals used, and **Refine** the methods used [7]. To conduct animal experiments an ethical approval is necessary. A regional animal ethics board issues the approvals. The board evaluates the potential scientific value from the experiment to the potential suffering of the animals. Personnel performing animal experiments are required to have a special education standardized by the Federation of Laboratory Animal Science Associations [8]. FELASA advocates responsible scientific conduct with animals in the life sciences with particular emphasis on ensuring animal welfare. When experiments include the death of the animal special emphasis is put upon the reduction of suffering.

The experiments included in the thesis were all conducted under balanced deep general anesthesia with the aim and effect of minimizing consciousness and pain sensations. The Animal Ethics Board in Uppsala issued the ethical approval (permit number C250/11).

The pig as a research animal. Rodents are the most widely used and studied experimental animals. However, the pig has certain features that make it

more suitable for inflammatory or infectious model experiments pertinent to humans. First, their immune system is highly similar to the human immune system (80%), whereas the mouse immune system is less similar (10%). Pigs react in a similar way as humans to endotoxin, whereas mice are highly resistant to endotoxin and tend to react with hypothermia to a challenge [9]. Second, there are many anatomical and physiological similarities between pigs and humans regarding organs such as heart, lungs, kidneys, spleen and central nervous system. In addition, the size of the pig allows for clinical monitoring including catheters and devices that are used for humans, such as endotracheal tubes and ventilators. Many surgical procedures can be performed in ways similar to procedures on humans, and the blood volume of pigs allows for repeated sampling [10]. Pigs have been used extensively in sepsis and ventilator-associated pneumonia models and contributed to the understanding of pathophysiology, pharmacology and efficacy of therapy [11,12].

The scientific downside of animal models relates to the translational relevance that comes from the experiments. The animals are often homogenous in regards to age, size and lack of co-morbidities, whereas the human target group in intensive care does not share these qualities. The experimental stimuli do not readily reflect the normal pathology found in humans. Examples pertinent to the thesis are intravenous administration of large quantities of bacteria or endotoxin to simulate sepsis, or inoculation of large quantities of bacteria into the lungs of the animals. And although animals share many features with humans, they are not human. The scientific upside is clearly the controlled situation where responses can be evaluated in correlation to an exact stimulus, interventions can be synchronized in time, and additional measurements like tissue samples are freely available at the experimental endpoint [13].

Anesthesia and ventilator-induced lung injury

Emergence of the smooth ride…and bumps in the road

The substances. In the year 1846, Dr. Morton had invented a new method of fitting false teeth that he hoped would make his dentistry business flourish. However, the method was so painful that few patients would undergo the procedure. He needed an analgesic substance more potent than **nitrous oxide** (N_2O), i.e. laughing gas, which was commonly used by dentists at the time. Dedicated his mission, he set out to test new substances on his pet gold fish, his dog, his dental assistants and himself - initially without great success. It was not until a purified form of **Sulphuric Ether** vapor was tested on a patient desperate to get rid of a painful tooth that events turned [14]. Business grew, and the word spread (not least from the dentists own mouth) all the way to the intimidating Chief of Surgery at Harvard, John Collins Warren. The skepticism among the surgeons was heavy after a recent failed demonstration with nitrous oxide. Warren was interested enough in a method like this to give it a try on a patient with a large vascular tumor of the neck. The scene was set for drama, and history was made with a triumphant Dr. Warren bellowing to the audience, "Gentlemen, this is no humbug!" [15]. Before this miraculous moment surgery without pain was unthinkable. Patients were either fully conscious or sedated with opium or alcohol. These circumstances had restricted the surgical interventions possible. The birth of anesthesia was a prerequisite for the practice of surgery to begin to evolve at speed [16].

Ether, as the new substance was called, was far from perfect – naturally. It was flammable, had a long induction time, an unpleasant odor and produced post-operative nausea and vomiting. An obstetrician named Dr. James Simpson set out to find a better anesthetic agent - a process that naturally included self-inhalation at dinner parties. The new agent that he promoted was called **chloroform** and was credited with qualities superior to ether such as higher potency, faster onset of action, pleasant odor and ease of use. It became widely used for decades until the side effects of hepatotoxicity and fatal cardiac arrhythmias when used with epinephrine were discovered. Several hundred anesthetic gases were tried and discarded during the first half of the 20th century. The reasons for rejection included explosive properties and high incidence of nausea and vomiting. By halogenating hydrocarbon compounds the flammability could be reduced. **Halothane** was a promising

agent introduced in the 1950´s with pleasant odor, high potency, low flammability and no toxicity (!). Until its' propensity to cause liver damage was discovered. Anecdotes of its use remain given from "the elder" anesthesiologists in coffee rooms still today. Modern halogenated hydrocarbon gases, such as the latest introduction of **sevoflurane** in the 1980´s, are widely used today.

Intravenous anesthesia was attempted at first in 1872 with **chloral hydrate**, and although successful the method did not gain popularity. Many substances were discarded because of side effects. In the 1930´s Dr. Lundy introduced the barbiturate **sodium thiopental.** He also introduced the term 'balanced anesthesia', suggesting that the use of plural anesthetic substances in lower doses could achieve the same result as a large dose of one substance but with fewer side effects. This view holds true still today. A drawback of the barbiturates was the cardio-depressant action leading to sudden deaths in trauma victims. The hallucinogenic **ketamine** discovered in the 1960's had special properties, as it could be administered both as intravenous and intramuscular injection. **Etomidate** discovered in the 1970's was cardio-stable even in high doses. **Propofol** introduced in in the 1970's, with short duration, no accumulation over time, anti-emetic properties and optimized for infusion became the jewel of intravenous anesthetics. It led to a paradigm shift in anesthesia, as volatile agents were no longer necessary. It is the most widely used agent of its' kind today and is often used in combination with other short acting analgesics in ambulatory surgery [15].

Muscle relaxants are not anesthetic agents, but interrupt the neuromuscular junction. The arrow poison substance **curare** was used clinically at first in the 1940's as an aid to surgeons where anesthesia alone did not relax the patient sufficiently. Many relaxants have been abandoned because of side effects. The steroid-based substances of today, such as **rocuronium,** maintain adequate safety [15].

Artificial breathing

Masks. The early anesthetics of the 19th century were delivered via simple measures such as masks or soaked rags over the nose and mouth. The delivery of the anesthetic agents was also dependent on the patients' own respiratory efforts under anesthesia. The emergence of artificial breathing is historically closely related to resuscitation, i.e. revival of the almost dead by correcting acute physiological disturbances, such as the lack of breathing [17]. The Greek physician Galen made advances in physiology in the second century A.D. He taught, wrongfully, that breathing caused the heart to beat. Nothing much happened in the understanding of ventilation or any other science in the next 1500 years to come, an era known as the Dark Ages. By

todays standards the progress made in the centuries to come involved some seriously gruesome animal experiments given that anesthesia was not even a word. In the 16th century, an anatomy professor named Andreas Vesalius questioned the teachings of Galen and pioneered the art of anatomical illustrations. He made the first reference to positive pressure ventilation when he described the resuscitation of animals by blowing air into collapsed lungs by way of a tube inserted into the trachea [18].

> "But that life may be restored to the animal, an opening must be attempted in the trunk of the trachea, into which a tube of reed or cane should be put; you will then blow into this, so that the lung may rise again and take air"

In the 17th century Robert Hook finally disproved Galen's theory that the movement of the lungs makes the heart beat. He cut holes in the chest wall and pleura of a dog and let a constant flow or air pass through the trachea and the lungs keeping the dog alive [19]. The reasons why people became pulseless and dead were not clear to physicians. In the 17th and 18th centuries a leading theory was that lack of stimulation led to the dead state. This belief led to interesting – but not successful - methods of resuscitation, such as throwing the unconscious patient onto a trotting horse, or blowing smoke up their rectum with a "fumigator". The late 18th century saw the discovery of oxygen by Priestly and Scheele, and its' importance to respiration was established by Lavoisier. The mouth-to-mouth resuscitation that had been in use for some time now suffered a temporary setback, as it was believed that exhaled air did not contain oxygen.

Negative pressure ventilators. The late 19th century saw the emergence of ventilators using sub-atmospheric pressure to suck air into the lungs. This mechanism is the principle applied in natural physiologic breathing, with the use of the diaphragm and the respiratory muscles of the thoracic cage to generate flow into the lungs. Alfred Jones devised a body-enclosing box with the head of the patient outside the box. He claimed in his patent application that the device [20];

> ". . . cured paralysis, neuralgia, seminal weakness, asthma, bronchitis, and dyspepsia. Also deafness . . . and when judiciously applied, many other diseases may be cured".

The first iron lung was made in France in the 1870's, but Drinker and Shaw in Boston made the model that enjoyed widespread use in 1929. It was used to treat polio victims suffering respiratory weakness [21]. The early devices were clumsy and displayed much impracticality in the care for patients. In the Childrens' hospital in Boston there were multi-patient chambers in use where nursing staff could attend to more than one patient at a time.

Positive pressure ventilators. In 1951 there was an international polio conference held in Copenhagen. The year after the city saw an enormous polio epidemic that quickly flooded the resources of the care facilities. Death rates were 80% in patients presenting with respiratory failure due to bulbar paralysis. Initially, due to the high carbon dioxide values measured in plasma, it was thought that patients died from acute kidney failure from an excessive viral load. A Dr. Ibsen with experience from Boston rightly diagnosed this condition as respiratory failure and succeeded to convince the management to try tracheostomy and positive pressure ventilation. Death rates dropped to 40% over-night [22]. As there were no positive pressure ventilators at the time some 1500 students in total ventilated the patients by hand. Many patients were for logistical reasons treated in one location, effectively establishing the first intensive care units.

The initial focus on ventilatory support soon expanded. Oxygenation failure was identified as blood gas measurements improved. The Acute Respiratory Distress Syndrome (ARDS) and the benefits of using positive end-expiratory pressure (PEEP) to reduce atelectasis were identified in the late 1960's [23]. The last decades of the century saw a shift from controlled ventilation in intensive care to ventilatory support forms, where the patients own respiratory drive and breathing efforts are synchronized with the ventilator.

Ventilator-induced lung injury

Mechanical ventilation was originally used on patients with normal lung function, such as polio victims or those undergoing anesthetized surgery. The ventilation of relatively healthy lungs required low insufflation-pressures. The technical development of positive pressure ventilators brought the possibility to apply higher positive pressures, which made them more efficient in providing gas exchange in already damaged lungs. It became obvious that damaged lungs were more sensitive to high-pressure ventilation, and the main problem was pneumothoraxes. Post mortems on polio victims coined the expression "respirator lung" that described diffuse alveolar infiltrates and hyaline membranes [24]. These complications were labeled "**barotrauma**", i.e. pressure induced trauma [17]. The mechanism of a tear in the junction between bronchiole and alveoli at high lung volumes, causing air to enter between the pleural sheaths was described in 1940's. In the 1960's, oxygen toxicity was regarded as a major threat. The foundations were animal experiments with higher mortality from 100% inspired oxygen, and complications in infants given the same. This reluctance to use oxygen led to the use of higher tidal volumes to correct blood gas abnormalities, and subsequently barotrauma and lung injury. Animal experiments concluded that intermittent high distending pressures could cause fatal lung damage [25]. Later experiments have refined the causal mechanism behind lung

damage to be the over-distension from high tidal volumes, labeled "**volutrauma**", and not pressure *per se* [26] (*Figure 1)*. Before the turn of the millennium "**biotrauma**" was coined as the biological consequences of injurious mechanical ventilation [27]. Injurious mechanical ventilation, i.e. allowing lung parts to collapse and reopen with each breath (cyclic atelectasis) and over distension of alveoli, will produce leakage of inflammatory mediators, bacteria and endotoxins to the systemic circulation. The theory is that these mediators subsequently affect organs distal from the lungs and eventually cause multiple organ dysfunction [28,29]. Apart from pure side effects on the lung itself, hemodynamic compromise from high intra-thoracic pressures is acknowledged [30].

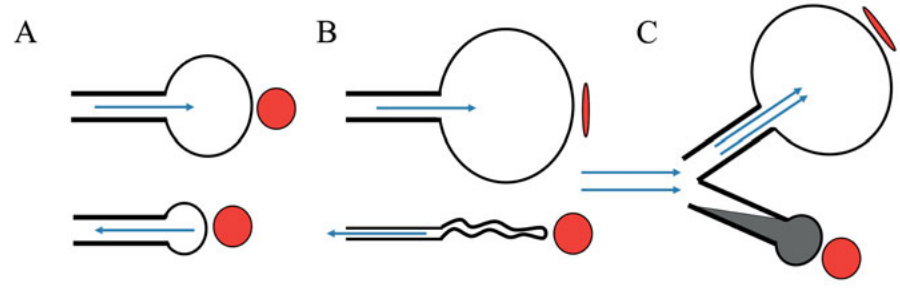

Figure 1. Schematic views of terminal bronchiolar, alveolar and blood vessel behavior in different states of ventilation. A) Ideal ventilation, alveolus open but not overstretched, blood vessel open, no atelectasis on expiration, B) overstretched alveolus, compressed blood vessel on inspiration and atelectasis on expiration (cyclic atelectasis), C) non homogenous lung with consolidated part forcing the inspiratory volume to overstretch the open alveolus and compress blood vessel. B and C contribute to ventilation/perfusion mismatch, inflammation, lung damage and biotrauma. Blue arrow airflow, red dot blood vessel.

Lung-protective ventilation

Healthy lungs during anesthesia and mechanical ventilation. During anesthesia the resting volume of the lung, i.e. the functional residual capacity (FRC), is reduced by loss of muscle tone. This promotes closure of small airways, gas resorption (faster with higher oxygen concentration) behind the closures and atelectasis formation. Ideally, the alveoli are open during respiration and the capillaries surrounding the alveoli carry flowing blood. However, the blood that flows past atelectatic areas cannot contribute to necessary oxygen and carbon dioxide gas exchange, a phenomenon called shunting [31]. Conversely, an over-stretched alveolus will compress the capillaries passing so that no blood flows past to mediate gas exchange *(Figure 1)*. These events are summarized as 'ventilation-perfusion mismatch' and are a large

part of the problem with ventilating injured lungs [15]. The atelectasis formation during anesthesia is reversible by easy measures. Although, there are many ways to perform a recruitment maneuver, the classic bagging consists of a sustained inflation pressure of 30 cmH_2O for 10 seconds. This will open most collapsed airways in healthy adults, and a pressure of 40 opens virtually all lung-healthy patients [32]. The application of a positive end-expiratory pressure (PEEP) increases the FRC and airway dimensions and prevents atelectasis of open airways. A PEEP of 10 cmH_2O has been shown to reopen collapsed lung tissue [33]. This effect is probably mediated by the elevation of the peak inspired pressure. In clinical practice atelectasis is formed by the anesthesia, reopened by the peak pressure, and counteracted by the PEEP. The optimal PEEP level during anesthesia is an open question. However, the use of a low level of PEEP, such as 5 cmH_2O, seems to be common [34].

Stress and strain are basic concepts of lung physiology. Stress is simplified the force on lung tissue per unit area from the applied pressure, and stress the change in lung volume to the resting volume during respiration [35]. These concepts are applicable on a micro-level and not easily used bedside using macro measurements of respiratory pressures and volumes. However, non-homogenous areas in damaged lungs may act as stress raisers, multiplying locally the applied global stress and strain. Consequently, the junction between healthy and damaged lung is especially vulnerable to applied pressures *(Figure 1)*. As the homogeneity of the lung decreases along with the aggravation of lung damage, the risk of inflicting ventilator-induced lung injury increases [36,37].

Protective ventilation. The concept of protective ventilation deserves a special introduction as it embraces many definitions, none of them trademarked. Breathing, ranking as the foremost prerequisite for life, certainly protects humans from dying within minutes. Mechanical ventilation can protect a patient from dying under circumstances when he or she cannot breathe. However, mechanical ventilation, be it harmful or gentle, does not protect from anything else. We talk about lung-protective ventilation and protective ventilation in a wider sense interchangeably. What the protective stands for in these cases is non-harmful or less harmful ventilation than another form of ventilation (often referred to as traditional) – it is relative! By applying protective ventilation we want to avoid or reduce the infliction of injury to the lung and the events triggered by it. The insight that the way we mechanically ventilate patients will affect other organs than the lung and patient outcomes is highly important. Protective ventilation has become the best practice standard in intensive care, but the role of protective ventilation remains uncertain for anesthetized patient undergoing major surgery [38]. The concept in different studies most often include both a reduced tidal volume and some level of PEEP, in effect a combined intervention. The influences of the separate parts of the combination remain abstruse.

Acute Respiratory Distress Syndrome has previously been named Respiratory Distress Syndrome, Adult Respiratory Distress Syndrome, or shock lung. The development and practice of protective ventilation is closely associated with ARDS, which is a form of non-cardiogenic pulmonary edema stemming from acute injury to the alveoli. In this state, mechanical ventilation can ease the work of breathing and alleviate the hypoxia. At best, the lungs are given time to heal, but mortality is high primarily from multiple organ dysfunction syndrome (MODS). Initially, x-ray shadows over the lungs were taken as homogeneous lung damage. The use of computer tomography refuted this picture and proved the damage to be non-homogeneous, with part of the lung damaged and part looking normal in aeration [36]. During mechanical ventilation the air takes the path of least resistance and flows to the normal areas, exposing them to the risk of over extension and barotrauma *(Figure 1)*.

The first international consensus definition of ARDS was in 1994 [39]. It included the acute onset of hypoxemia (arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) < 200 mmHg), bilateral infiltrates on chest radiograph, and no evidence of left sided heart failure measured by left atrial pressure. Acute lung injury (ALI) was defined similarly but with less severe hypoxemia, < 300 mmHg. The latest definition from the ARDS task force in 2012 has corrected some of the shortcomings of the old definition. ALI was taken out, the timing of onset was defined to within one week of clinical insult, the chest imaging criterion was simplified, and the levels mild, moderate and severe ARDS were added according to decreasing oxygenation criteria [40]. Each progressive level of ARDS is associated with increased mortality and length of hospital stay.

A traditional target during mechanical ventilation has been to normalize blood gases. However, in 1990 a study that reduced tidal volumes at the expense of higher carbon dioxide values produced lower mortality [41]. During the 1990's the attention was turned to reduction of tidal volumes. Four randomized trials showed conflicting results. One study showed reduced mortality from reduced tidal volumes, but three did not show any difference [42–45]. It was not until the turn of the millennium when the Acute Respiratory Distress Syndrome Network published a milestone multicenter trial proving the benefit of reduced tidal volumes [46]. The trial compared what was called "traditional" tidal volumes of 12 mL x kg^{-1} and a maximum plateau pressure of 50 cmH_2O, with a protective protocol of corresponding 6 mL x kg^{-1} and 30 cmH_2O. Thus, the protective regimen was a combination of reduced tidal volume and reduced plateau pressure, and was designed to avoid excessive stretch of the alveoli. The PEEP levels used (5-24 cmH_2O) were connected to the inspiratory oxygen fraction required (0.3 – 1), and did not differ between the two groups. The trial was stopped after the inclusion of 861 patients because the mortality was lower in the low tidal volume group (31.0 vs. 38.9%). The study had enormous impact clinically, a rare

occurrence. What constituted traditional tidal volumes turned into historically obsolete volumes after the publication. At least that is true for ARDS treatment in the intensive care setting.

Protective ventilation in non-ARDS patients. The use of low tidal volumes outside of ARDS was not well studied. The area includes any patient with initially healthy lungs that for some reason requires mechanical ventilation. Examples are patients suffering acute intoxications and unconsciousness, intra-cranial hemorrhages, pneumonias with respiratory fatigue, and those undergoing any form of surgery under general anesthesia. One study addressed the issue of protective ventilation on intensive care treated patients without the criteria for lung injury at the start of mechanical ventilation (6 mL x kg^{-1}, compared to 10 mL x kg^{-1}) [47]. The data suggested that traditional tidal volumes in these patients correlated with development of lung injury and with higher sustained cytokine production. Large tidal volumes and high peak airway pressures used at the initiation of mechanical ventilation were identified as independent risk factors for the development of ARDS [48]. Animal experiments offered additional supporting evidence on the issue. It was shown in rabbits that ventilation, which under normal circumstances was non-injurious to the lung, turned harmful under the influence of general inflammation induced by endotoxin [49]. The clinical implications of these findings point directly at situations such as emergency surgery and perioperative sepsis where systemic inflammation is likely to be found. Other animal experiments provided proof of concept that protective ventilation not only reduced the negative effects on lungs, but additionally to other organs [28]. Studies had now shown that protective ventilation reduced serum cytokine levels during lung injury [50], reduced end-organ dysfunction [51], and reduced mortality in ARDS [46]. The big question was certainly if protective ventilation could reduce the development of multiple organ dysfunction, and if so how [52]. Inflammatory mediators, bacteria and endotoxins were proven to escape into systemic circulation through leaking lung epithelia during ARDS [53,54]. These facts support the biotrauma hypothesis posed by Slutsky. It describes a mechanism by which spread of inflammatory mediators from the lungs during ventilation have subsequent effects on end-organs resulting in MODS [27].

Protective ventilation in the perioperative period. Protective ventilation regimens have been tried in different surgical settings with differing results, and its use remains controversial [55]. Already in 1990 before the renewed interest in the subject, a randomized trial on postoperatively ventilated patients indicated the safety of using low tidal volumes as it led to less pulmonary infection, shorter duration of intubation and hospital stay. Most interestingly, the reduced levels of oxygenation in the low tidal volume

group were clinically irrelevant to outcome [56]. Esophagectomy-patients exposed to one-lung ventilation showed lower inflammatory response, improved lung function and earlier extubation when ventilated with reduced tidal volume and PEEP [57]. There have been negative trials performed on patients in general, major thoracic and abdominal surgery. The studies compared different tidal volumes with or without PEEP and could not find any inflammatory differences within a timespan of a few hours [58,59]. A more recent large cohort study on cardiac surgery patients clearly identified the use of tidal volumes of more than 10 mL x kg^{-1} as a risk factor for organ dysfunction and prolonged intensive care [60]. The multicenter trial IMPROVE selected patients at high risk for pulmonary complications after abdominal surgery and found the use of protective ventilation associated with improved clinical outcomes and reduced health care utilization [38]. The risk profile for patients can be estimated based on age, co-morbidities and functional capacity [61].

Summarily, the use of protective ventilation seems to have a place in patients with high risk for postoperative complications undergoing surgery of a certain magnitude.

Immune system and inflammation

The following account has no claim on being all covering, but merely addresses selected issues pertinent to the experiments included in the thesis and their translational relevance.

The experiments are described as inflammatory models. Inflammation was stimulated in three basic ways - by **surgery**, by **endotoxin infusion** and by **inoculation of bacteria** in the lungs. Additionally, the different inflammatory stimuli were used in combinations in the experiments. When the different stimuli are separated in time we call them multiple hits (first hit the surgery - second hit the complication of sepsis – third hit the ventilator-associated pneumonia etc.). Inflammation and infection are highly important to human life and to medical clinics. The **immune system** comprises biological structures and processes within an organism that protect against disease. **Inflammation**, an early response of the immune system cells is a vast area. It is the response of the immune system to damage of cells and tissue caused by microbial pathogens or chemical or physical insults [62]. Celsus described the classical signs of inflammation of heat, pain, redness and swelling in the first century. Molecular agents secreted from immune cells already present in the tissue, predominantly mast cells and macrophages mediate the symptoms. Release of histamine causes vessels to dilate and leak fluid, and prostaglandins sensitize sensory nerve-endings producing pain. Inflammation helps to contain and remove the threat and is part of the healing process. However, inflammation that turns chronic can cause debilitating injury, organ damage, or cancer [63]. Localized inflammation can turn to systemic spread with acute devastating effect on the bodily functions causing imminent death. Patients with sepsis that are treated in intensive care exemplify the latter. Inflammatory responses can be **quantified** with the use of physiological variables such as fever, and by inflammatory markers in blood such as cytokines. Several important cascade networks such as the kallikrein-kinin, the coagulation, the fibrinolysis and the complement systems are activated during general inflammation [64].

Systems, cells and messengers of inflammation

The immune system can be branched into the **innate** system, and the **adaptive** system. Simply put, the innate system is a basic, unspecific, early re-

sponse system that requires no prior sensitization to pathogens except that offered by evolution. The adaptive system is initially slower and requires sensitization to form anti-bodies against specific pathogens, a process utilized in the vaccination mechanism where immunity in a controlled way is gradually increased by repeated exposures to the pathogen [64]. For all practical purposes, the problems of the inflammatory storm that we have tried to model after clinical scenarios in our experiments depend on the innate immune responses. The cells of the innate immune system are first line responders to irritation or infection, and interact with hormones and soluble mediators. The initiation of acute inflammation comes from cells already present in all tissues. These include resident macrophages, dendritic cells, histiocytes, Kupffer cells and mastocytes. Contained on their surface or within the cells are **pattern recognition receptors** (PRRs), which recognize molecule sequences that are broadly shared by pathogens but distinguishable from host molecules [65]. The hostile patterns are collectively referred to as pathogen-associated molecular patterns (PAMPs). At the onset of an infection, burn, or other injuries, the immune cells undergo activation (one of their PRR recognize a PAMP) and release inflammatory mediators responsible for the clinical signs of inflammation. Chemical factors produced during inflammation include histamine, bradykinin, serotonin, leukotrienes and cytokines.

Macrophages are the mature, tissue-migrated form of monocytes found in blood. They have a pivotal role in early immune reactions where they sense and digest dangerous matter, present it to other immune cells, secrete pro-inflammatory mediators and recruit other immune cells from circulation to the tissue. Gram-negative bacteria are especially potent activators of macrophages. Macrophages take an active part in resolving the inflammation by consuming dead tissue and white blood cells and remove them from the body. They are found abundantly in different tissues where they specifically adapt [66]. Examples of macrophages relevant to the experiments are alveolar, pulmonary, intestinal and splenic macrophages, Kupffer cells of the liver and glial cells of the brain.

Neutrophils are the storm troopers of the immune system. They are the most abundant white blood cell in the circulating blood, and normally not present in healthy tissue. Upon inflammation, mediators such as histamine dilate blood vessels and make them leak fluid to the tissue. The neutrophils migrate through the leaking vessel wall and move toward a gradient of chemo-attractant molecules secreted mainly from macrophages. The neutrophils phagocytize pathogens and kill them, whereupon they self-destruct in a process called apoptosis. Additionally, they secrete toxic substances that destroy pathogens and tissue alike [67]. Dead neutrophils and bacterial debris make up pus in the wound. A part of the cohort of neutrophils is loosely attached to the vessel walls at any given moment.

Toll-like receptors (TLR) are a family of pattern recognition receptors, which are expressed on cells of the innate immune system and endothelia [68]. They recognize specific PAMPs and initiate an inflammatory reaction upon stimulation. The TLR2, 4 and 5 are of special interest as they recognize pili, endotoxin and flagellin respectively - all constituent parts of the cell wall in the bacterium *Pseudomonas aeruginosa* used in the thesis experiments.

Endotoxin *(detail in Figure 4)* consists of an outer variable sugar part called the O-antigen, a connecting core part, and an inner lipid A part that carries the central biological effect. Endotoxin is shed in small amounts during bacterial multiplication, i.e. growth. Larger amounts are shed when host cells, antibiotics or both kill bacteria. Exposure to endotoxin is a very potent way to trigger an inflammatory reaction from the innate immune system, and from macrophages in particular. Endotoxin can be purified for experimental purposes and has been widely used in models that simulate the clinical effects of gram-negative sepsis [9]. We have used purified *Escherichia Coli* endotoxin in the experiments.

Endotoxin tolerance (ET) was described in the 1960's as a blunted inflammatory response to a repeated dose of endotoxin. Animals that were pre-exposed to a low dose of endotoxin exhibited reduced mortality from a subsequent presumed lethal dose [69]. There has been considerable interest in the phenomenon and its relation to inflammatory responses during different stages of sepsis. Early and late ET are described as having different mechanisms. The early form is a nonspecific refractory state of responsiveness of immune cells, and the late form is antibody mediated [70]. The phenomenon became measurable when cytokine analyses were introduced, and correlated with levels of tumor necrosis factor alpha (TNFα) and interleukin 1 [71][49]. Although ET modifies many responses, it has become almost synonymous with the reduction in endotoxin stimulated TNFα levels. There is lack of a clear definition of ET in regards to molecular processes involved. Many other agents than endotoxin can induce the same reaction. Surgery can induce ET, possibly by the release of anti-inflammatory IL10 [72]. The presence of ET in intensive care patients with sepsis has been correlated with relatively poorer outcome [73].

Cytokines are small soluble peptides (larger peptides are called proteins) that work through receptor interaction. Their function is to mediate immune responses, to act as catalysts to enhance or to shut down immune cell activity and to recruit other cells to the site of injury. Most nucleated cells secrete them. Endothelial and epithelial cells and a variety of cells of the innate immune system - such as macrophages, mast cells, stromal cells and leukocytes - are potent producers of cytokines. The complexity of cytokine interaction is vast and not fully elucidated. Based on their main action cytokines are labeled either **pro-** or **anti-inflammatory** [74]. Both sorts are released at the same time at the initiation of an inflammatory stimulus, but the balance is

tilted towards pro-inflammation in early sepsis. A certain cytokine can be expressed by different cells and can in addition have different functions. The term 'pleiotropic', meaning multifunctional, is used for many cytokines. It is largely unknown to which extent cytokines have functions in systemic circulation during health, as systemically measurable levels of cytokines are low in health. Although plasma levels of cytokines are related to systemic inflammatory states, relatively little is known about where these cytokines are produced. Evidence suggests that it is tissue migrated macrophages or endothelia rather than cells in circulation such as monocytes that are the biggest net providers of cytokines during sepsis [75]. The reason that cytokines are measured in systemic blood and not in the organs is of course a matter of availability and risk. We cannot remove patients' organs just to measure the expression of a biomarker. The second paper in the thesis deals to some extent with these issues. In all experiments we have used three widely described cytokines as markers of inflammation; the pro-inflammatory TNFα and interleukin 6 (IL6), and the anti inflammatory IL10 [74].

Anti-cytokine efforts. The unmistakable correlation between cytokines and systemic inflammation has led to efforts to manipulate cytokine activity to achieve clinical effects. A positive example is the use of TNF-alpha blockers to treat autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease [76]. With respect to intensive care there are experimental and clinical efforts to reduce the cytokine storm that accompanies the early stages of sepsis by blocking specific cytokines such as TNF-alpha, or wash them out of the system with the use of specific filters in dialysis machines [77,78]. The results remain debatable.

Nitric oxide (NO) is a gaseous radical that is involved in a great number of variable biological functions in both bacteria and mammalian cells [79]. In the 1980´s it was first identified as **endothelial-derived relaxing factor**, which indicates one of it´s trademark biological effects of vasodilation. It has since been identified in the regulation of platelet function, nerve transmission, metabolic control, and mitochondrial function to name a few areas. It is used as a coronary vessel dilator in medical treatment by way of NO-donors in ischemic heart disease and for systemic pre- and afterload reduction in heart failure. Inhaled NO can be used to dilate pulmonary vessels to reduce states of pulmonary hypertension [80]. The NO molecule is highly unstable with a short half-life and predominantly has autocrine or paracrine function, i.e. short range of action. Hence, the measurement of NO production is usually done by route of its by-products nitrite (NO_2^-) or nitrate (NO_3^-). Nitrate, the more common of the two, has a half-life of approximately 5 hours in circulation and is excreted by the kidneys [81]. We have measured nitrite/nitrate concentrations in urine in our experiments and used it as a marker of NO production. Two ubiquitous constitutively expressed enzymes, nitric oxide synthases (NOSs), and one inducible enzyme (iNOS) regulate the production of NO in the body. Inflammatory mediators such as endotox-

in and cytokines induce transcription of iNOS, which results in enhanced levels of nitrite and nitrate during systemic inflammatory states such as sepsis. Macrophages and neutrophils that are activated by inflammation contribute in a major way to NO production in the tissue [79]. The NO production affects functions such as chemotaxis, adhesion, apoptosis and bacterial killing. Additionally, it reduces the tissue harm inflicted by the superoxide ions used to kill bacteria by way of accepting an electron [82]. Elevated production of NO during sepsis has been associated with mitochondrial dysfunction, energy depletion and organ failure [83], but there are also evidence of beneficial outcomes from the use of NO donors in sepsis [84].

States of systemic inflammation

Systemic Inflammatory Response Syndrome

In 1991, there was a sepsis definitions consensus conference with the task to develop clinical parameters to aid the early inclusion of patients in sepsis trials [85]. Previously, the terminology surrounding sepsis had had suffered from lack of clarity. This lack was believed to have hampered research comparability and development of possible therapies. The acronym **Systemic Inflammatory Response Syndrome** (SIRS) was introduced, and although it encompassed more disease states than sepsis it founded a basis for clinical trials during the next 20 years to come [86]. The definition of SIRS applied to adults over 18 years of age and included two or more out of four, rather unspecific condition criteria that shared the advantage of being readily available at most treatment facilities;

1. Temperature >38°C or <36°C
2. Heart rate >90 beats per minute
3. Respiratory rate >20 breaths per minute or PaCO2 <4.3 kPa
4. White blood cell count >12000/cu mm, <4000/cu mm, or >10% immature forms.

SIRS can be initiated by several different stimuli such as trauma, burns, pancreatitis or infection from different pathogens such as bacteria, viruses, fungi etc. (*Figure 2*). Infection correspondingly can occur without the presence of SIRS. **Sepsis** is present when infection is the cause for SIRS. Critique has been presented of the overly sensitive definition of sepsis and the lack of usefulness for bedside clinicians. In 2001, a new conference could not find any evidence to support a change the definition [87].

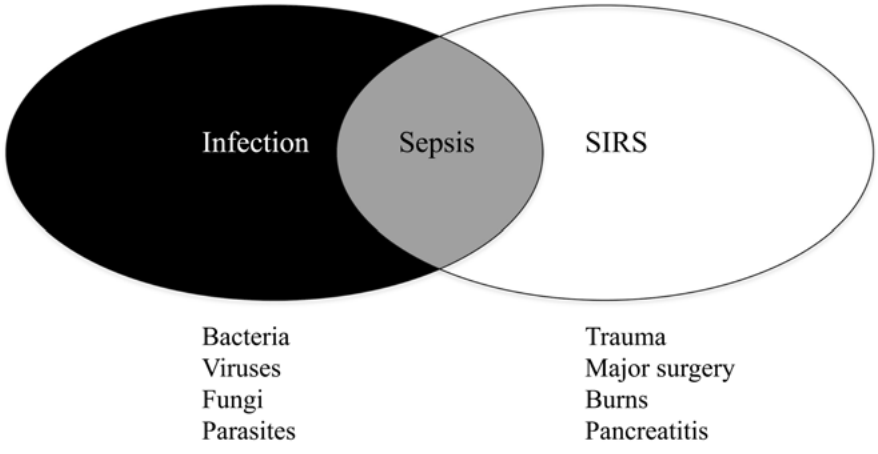

Figure 2. Schematic description of the relation between infection, sepsis, and the Systemic Inflammatory Response Syndrome (SIRS). Common causes are listed below.

Sepsis

Sepsis is the generalized systemic response to an infection. The inflammation that would be functional if contained in the tissue now occurs in the blood vessels. A common clinical picture of sepsis is that of fever, leukocytosis, tachycardia, difficulty of breathing, hypotension and different severity of organ dysfunction. There is a progression in disease severity to more advanced forms of sepsis, i.e. **severe sepsis** and **septic chock**. Severe sepsis indicates hypoperfusion, organ dysfunction or hypotension. These conditions are exemplified by increased lactate production, deterioration of mental status, diminishing urine production, coagulation disorders and systolic blood pressure reduction to below 90 mmHg [64]. Septic shock ensues when adequate fluid resuscitation (a subject of considerable controversy) does not restore blood pressure or resolve the hypoperfusion, necessitating vasopressor therapy. Patients with the two more severe forms of sepsis are commonly treated in intensive care facilities. The mortality from sepsis has historically been high, and still is the leading cause of death in intensive care [88]. Death from sepsis is as common as that from myocardial infarction in hospitalized patients [89]. Sepsis is the largest cause of **Multiple Organ Dysfunction Syndrome**, a state that was described in the 1970´s and was called by many names until the consensus conference in 1991. Before the intensive care era patients died before they could develop MODS. MODS is commonly seen from major insults to the body that do not primarily affect the organs failing, in effect all conditions that initiate SIRS. This organ dysfunction has been proposed to have protective purposes for the host as the cells of the organs do not die, but merely shut down their metabolic activity to a minimum and

enter a hibernating state [90]. Examples of this are that patients who survive sepsis with renal failure and intensive care dialysis rarely end up with end-stage renal failure, and that organs from non-survivors often show minimal cell damage [91,92]. At he heart of the matter lays the function of the mitochondrion, the cells power plant [83]. The mitochondrial activity in producing energy accounts for more than 90% of total oxygen consumption. The organ hibernation is likely originating from the effects on mitochondria inflicted by cytokines and NO, both produced in excess during sepsis [93,94].

The last decades have seen intense activity in sepsis-related research with aims to develop molecular diagnostics and to identify biological markers and potential targets for therapy [95]. Although many promising angles of interception were identified, many of the large studies of the last 20 years that showed initial promise (i.e. activated protein C [96], early goal-directed therapy [97], low-dose cortisone [98]) later have failed to confirm the findings [99,100]. In effect, the efforts to refine the diagnosis of sepsis from the basic physiological descriptors to biological markers have failed so far. Critique has been formulated such,

> "It makes no sense to use twenty-first century technology to develop drugs targeted at specific infections whose diagnosis is delayed by nineteenth-century methods" [62].

On the brighter side, a recent large study from Australia-New Zealand ranging over a decade indicated a significant drop in sepsis-related mortality from 35 to 18% [101]. With the magic bullet to treat sepsis still to be found it is tempting to credit the lower mortality to a slow train moving – the general improvement in the standard of care. Large-scale clinical initiatives like the Surviving Sepsis Campaign have taken evidence-based management strategies from bench to bedside [102]. Especially, the early treatment bundles applied during the first pivotal hours after diagnosis may have helped to reduce mortality [103]. The incidence of post-operative sepsis is has in a large study increased at the same time as the mortality has decreased [104].

It has been recognized that the early intense immune response to sepsis not solely is pro-inflammatory, but also initiates a counter reaction dubbed **Compensatory Anti-inflammatory Response Syndrome** (CARS) [105]. Patients that survive the early stages of sepsis can develop a state of hypo-inflammation or immunoparalysis where they are susceptible to reactivation of latent viruses and new infections (*Figure 3*). Bacteria found in these super-infections are not commonly found in immune-competent patients [106].

The experiments of the thesis that utilize endotoxin induced SIRS to simulate sepsis are rightly not **sepsis models**, as there is no infection present. However, endotoxin is widely used and very effective in eliciting the desired inflammatory response. Other models of sepsis could be exemplified with the cecal ligation puncture procedure (i.e. intra-abdominal spread of bacteria

from the intestine which produces multimicrobial peritonitis), and infusion of bacteria into the blood stream [107].

The surgical stress response

Preparatory surgery was a necessary part of all the experiments so that measurement devices and sample catheters could be used. Additionally, the surgery part made the models relevant to a common clinical scenario where sepsis complicates the postoperative period. This complication is mainly found after major surgical interventions [108].

The surgical stress response, or trauma response is found in all vertebrates. It is a predictable series of events that, likely, function to maximize the healing potential [109]. A biphasic immune, inflammatory and metabolic response to injury was observed already in the 1930´s by Cuthbertson [110,111]. The first phase was called **ebb**, in latter days better known as shock, and directed the organism to immediate survival. Peripheral vessels constrict and blood and substrates are directed towards essential organs, the metabolic expenditure is set to a minimum and salt and water are conserved. As shock in most cases is a treatable state, the bodily reactions turn to the next phase when survival seems probable. The subsequent **flow** phase is a state of hypermetabolism, where muscle proteins are degraded to produce amino acid substrates for new protein production and sugar to feed leukocytes. Teleologically, it has been proposed that this inflammatory reaction has a nutritional reason and that all systemic inflammatory reactions share the common evolutional rational for survival of the host [112]. Delivery of nutrients to injured and avascular tissue requires leakage from the blood vessels producing edema. When revascularization occurs the process is reversed and fluid is resorbed from the tissue. In the event that the flow phase does not resolve, a state of constant hypermetabolism can lead to MODS and death. Inflammatory cells such as macrophages and neutrophils are part of all phases of wound repair [113].

Indeed, the ability to withstand the increased metabolic demand in the post injury period is related to survival [114,115]. The magnitudes of the different responses reflect the degree of surgical stress [116] (*Figure 3*).

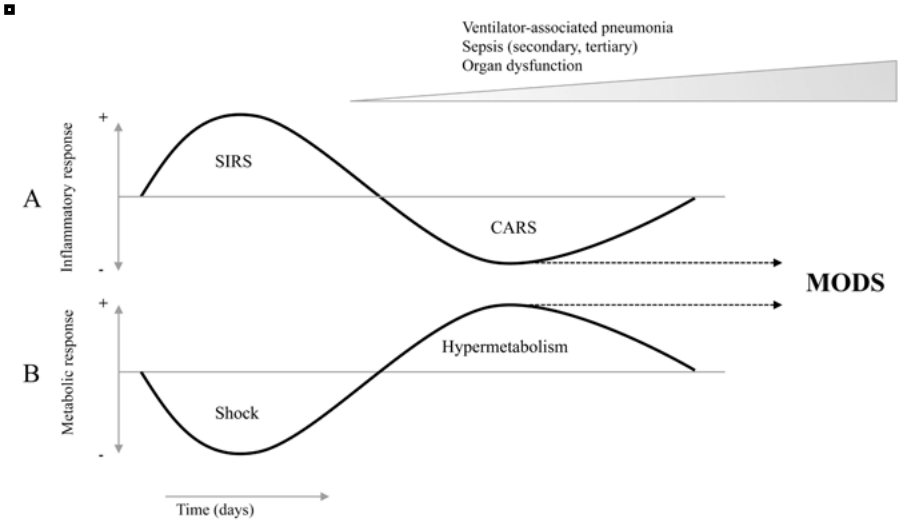

Figure 3. Schematic figure over the A) net inflammatory and B) net metabolic responses to major inflammatory stimuli such as sepsis or trauma. Black lines indicate the natural biphasic responses that eventually restore homeostasis (gray line). Failure to return to homeostasis may result in Multiple Organ Dysfunction Syndrome (MODS) and death. Systemic Inflammatory Response Syndrome (SIRS), Compensatory Anti-inflammatory Response Syndrome (CARS). The risk for complications increases in the later phases.

Pulmonary infections

Ventilator-associated pneumonia

Different definitions of ventilator associated pneumonia (VAP) have been proposed over the years, but none has been universally accepted [117]. Conceptually, VAP is an inflammation of the lung parenchyma by a pathogen that was not present at the time of initiation of mechanical ventilation. A common definition has been that the infection has to occur more than 48 hours after the initiation of ventilation. The time until the onset of infection has been used to divide VAP into early and late categories. The early onset VAP occurring within 4 days is usually more easily treated by antibiotics and carries a better prognosis for the patient. Common pathogens are *Staphylococcus aureus*, *Streptococcus pneumonia* and *Haemophilus influenzae.* The late onset VAP after 5 days involves multi-drug resistant pathogens to a higher degree, and has severe impact on outcome for the patient [118]. Common pathogens are *Pseudomonas aeruginosa*, *Acinetobacter*, *Enterobacter spp*., or methicillin-resistant *S. aureus*. VAP is the most common acquired infection in intensive care, and the second most common hospital acquired infection after urinary tract infections in a large survey [119]. The

incidence varies over a wide range depending on definitions, locations, patient characteristics and antibiotics policy. The attributable mortality from VAP is a matter of debate, but expert opinion estimates that 20-30% of patients diagnosed die from their infection [118]. *P. aeruginosa* has been identified as the most common pathogen causing VAP in patients that require a tracheostomy for extended time in mechanical ventilation [120]. Additionally, *P. aeruginosa* mediates an excess morbidity and mortality in this patient category when compared to other pathogens [121].

The risk factors for acquiring VAP are all related to severity of illness and time in intensive care, i.e. reintubation, tracheostomy, multiple central venous line insertions, enteral feeding through a nasogastric catheter, antacid use and coma [122]. The source of the pathogens causing VAP can be either from outside or from within the body. Gastric and oropharyngeal colonizations are intrinsic routes, whereas contaminated humidifiers, suction catheters, bronchoscopes or dirty hands are extrinsic [123].

The diagnosis of VAP is difficult. In practice, it is often made from the occurrence of new infiltrates on radiography together with (2 out of 3) clinical signs, i.e. fever, leukocytosis or leukopenia, and purulent secretions from the trachea [124]. An autopsy study on acute lung injury patients showed that clinical diagnosis alone had a high frequency of both false positives and false negatives [125]. Microbiological confirmations and quantifications of bacteria from suspected infections are done by methods such as blind bronchial sampling, bronchoalveolar lavage and protected specimen brush sampling. The outcomes of bacterial cultures made from these methods depend heavily on the quality of the sample, and false negatives are common [117]. Antibiotics are needed to treat VAP, but large efforts have been put into the application of preventive measures in care bundles – some parts more easily applicable than others. These include amongst others implementation of hand hygiene, minimizing time in mechanical ventilation, semi-recumbent position, and the avoidance of reintubation, tracheostomy, corticosteroids and stress ulcer prophylaxis [126].

Summarily, VAP is hard to define, hard to diagnose, hard to treat, costly and deadly. It should be avoided.

Bacteria

Escherichia coli (*E. coli*) is relevant to the experiments in the thesis as the source of purified endotoxin used to generate a general inflammatory response (*E.coli*: 0111:B4 (Sigma Chemical Co., St Louis, MO, USA)). It is a Gram-negative bacterium that is necessary for the normal digestive process and it innocuously colonizes the colon. Additionally, it is an important pathogen for humans with an array of infections to its name, ranging from benign common cystitis to bloodstream infections causing SIRS (*E. coli* sepsis). It is a common pathogen derived from emergency abdominal surgery and from

translocation from the intestinal mucosa in states of ileus. It possesses four different surface antigenic structures (O, K, H, F) that convey different qualities pertinent to its survival and to its pathogenicity [127]. The O-antigen is the polysaccharide part of endotoxin (*Figure 4*).

Pseudomonas aeruginosa (*cover*) serotype O3 is used in Papers III and IV of this thesis. *P. aeuruginosa* is ubiquitous in nature. In humans, it is an opportunistic pathogen commonly found in patients that are immunosuppressed from cortisone, under chemotherapy or suffering prolonged critical illness. Colonization occurs at an early age in patients with cystic fibrosis due to reduced mucus clearance [128]. It is an aerobic, rod-shaped, Gram-negative bacterium with a unipolar flagellum and surface structures (pili) specialized in attaching to epithelia. The flagella and pili act as ligands to Toll-like receptors on scavengers. Mutants that do not express these are less immunogenic and have a higher chance to persist. Bacteria found in colonized individuals therefore have different characteristics than those found in patients with acute infections. Most strains excrete a blue-green pigment, procyanin, with proinflammatory properties. The pili are essential for initial attachment to airway epithelium. The flagellum is for motility until colonization has occurred. A very large genome gives potential for adaption to circumstances that would be inhospitable to other species. The expression of the flagellum is lost in synchrony with the start of **biofilm** production. The production of biofilm is used as protection from anti-bodies and attacking phagocytes in the airways. The bacteria have a developed cell-to-cell signaling system that coordinates the expression of genes pertinent to adaption and even manipulation of the host inflammatory system.

A variety of virulence factors such as exotoxins and enzymes are expressed. The **type III secretion** system is the major determinant of virulence. It requires pili-epithelial interaction and allows for injection of toxins into host cells. This secretion is especially important in acute invasive infections [129,130]. Exotoxin A mediates tissue damage and cell necrosis and facilitates the dissemination of the infection. Macrolide antibiotics have reductive effects on type III secretion and biofilm formation, even though they do not possess bactericidal action [131]. The blocking of type III secretion in animal sepsis models has lead to improved survival [132]. Isolates expressing type III secretion from patients with ventilator-associated pneumonia are associated with worse outcome [133]. The polysaccharide O-antigen side chain of pseudomonas was used for typing before genomic methods were in use. In total, the endotoxin from *P. aeruginosa* is less immunogenic and elicits lower cytokine responses from macrophages than *E. coli* endotoxin [134] (*Figure 4*).

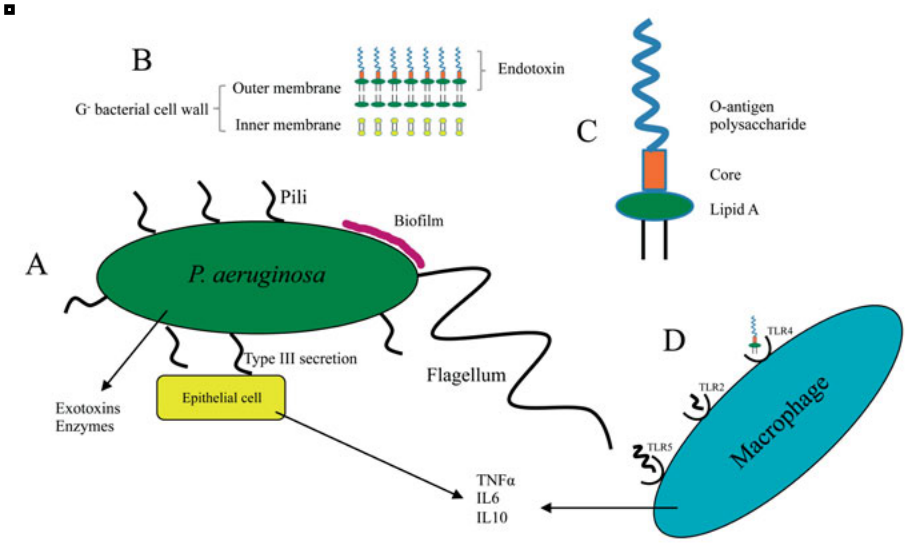

Figure 4. Schematic figure of interactions between *P. aeruginosa* and immune cells, exemplified by an epithelial cell and a macrophage. A) Bacterium with virulence factors, B) the G^- bacterial cell wall, C) constituent parts of the endotoxin molecule, D) macrophage with Toll-like receptors (TLR), bacterial ligands and resulting cytokine excretion, TLR2 (pili), TLR4 (endotoxin), TLR5 (flagellin).

Aims

I The study aimed to investigate whether protective ventilation - with low tidal volume and high positive end-expiratory pressure - would attenuate the systemic inflammatory response and organ dysfunction in a porcine model of postoperative sepsis.

II The study aimed to investigate whether protective ventilation would affect the organ-specific immune response in a porcine model of postoperative sepsis.

III The study aimed to investigate whether protective ventilation would affect the growth of *Pseudomonas aeruginosa* in lung tissue and the development of lung damage during experimental pneumonia in a porcine model.

IV The study aimed to investigate whether mechanical ventilation, with and without systemic inflammation induced by endotoxin, would alter the immune response to a pulmonary *Pseudomonas aeruginosa* challenge and affect the bacterial growth in lung tissue and the development of lung injury.

Methods

The same basic methodology was used in all experiments regarding anesthesia, preparatory surgery and laboratory blood sample handling. Differences will be described with respect to each experiment. Details on methods are summarized and further detail can be found in the respective Papers I-IV.

Anesthesia

The animals were induced in the transport box by an intramuscular injection consisting of tiletamine 3 mg x kg^{-1}, zolazepam 3 mg x kg^{-1}, xylazine 2.2 mg x kg^{-1} and atropine 0.04 mg x kg^{-1}. Within approximately three minutes the animals were unconscious and put on the operating table. An intravenous catheter was placed in an auricular vein. Fixed doses of morphine 20 mg and ketamine 100 mg were given before the first surgical incision. Anesthesia was maintained during the whole experiment by a continuous infusion of sodium pentobarbital 8 mg x kg^{-1} x h^{-1}, morphine 0.26 mg x kg^{-1} x h^{-1}, with the addition of the muscle relaxant pancuronium bromide 0.48 mg x kg^{-1} x h^{-1} – all dissolved in 2.5% glucose solution. Due to manufacturing reasons the use of pancuronium bromide was changed to a separate infusion of rocuronium at an initial rate of 2 mg x kg^{-1} x h^{-1} in Papers III and IV. During all experiments, the need for additional analgesia or deepening of anesthesia was met with fixed doses of morphine 20 mg and ketamine 100 mg. The total background fluid load was 15 mL x kg^{-1} x h^{-1} in papers I and II (anesthesia infusion 8 + Sodium Chloride 0.9% 7 mL x kg^{-1} x h^{-1}). In papers III and IV it was 10 mL x kg^{-1} x h^{-1} (anesthesia infusion 8 + Ringer´s Acetate 2 mL x kg^{-1} x h^{-1}).

Preparatory surgery

The surgery lasted approximately 1,5 (I, II) and 1 (III, IV) h and parts are presented in sequence. Surgical areas were soap washed and disinfected with alcohol before incisions. **Tracheostomy** was performed by skin incision, blunt dissection, sharp opening of trachea and tracheal intubation with a tube size 8. Incisions were made on both sides of the neck for exploration and dissection of vessels before insertion of catheters. On the right side catheters

were inserted into a neck **artery** 5F, into a **central vein** and into the **pulmonary artery** (Swan-Ganz, 7F). The two latter were accessed via the external jugular vein. On the left side a catheter was inserter in a cranial direction via the internal jugular vein to reach a location corresponding to the **jugular bulb**. Additionally on the left side, an introducer was inserted into the external jugular vein, whereupon a Swan-Ganz catheter was placed under fluoroscopic guidance in the **hepatic vein**. A 20 cm skin incision was made in the left subcostal region, followed by blunt dissection of muscle layers, penetration of the peritoneum and evisceration of the spleen. An arterial catheter was inserted over a guide-wire into the splenic vein approximately 15 cm to reach the **portal vein**. The location was confirmed by fluoroscopy. The spleen was then put back into the abdomen. The urinary bladder was catheterized by a small midline cystotomia. All skin incisions were sutured.
In Papers III and IV, no catheters were placed on the left side of the neck (no jugular bulb, no hepatic vein).

Physiological measurements

Physiological variables were recorded regularly in all experiments, although not every hour during the first 24 h in experiment IV that extended over 30 h. Circulatory variables were mean arterial pressure, cardiac output by the thermodilution technique, pulmonary capillary wedge pressure and mean pulmonary arterial pressure. Respiratory variables were proximal airway pressures, respiratory volumes and (in I) functional residual capacity using the SF6 inert gas method [135]. Additional variables were temperature and urine output.

Laboratory measurements

In all experiments blood samples from the artery were used for measurements of blood gases, complete blood count, inflammatory cells and cytokines (TNFα, IL6 and IL10). The organ specific catheters in papers I and II (i.e. jugular bulb, portal vein and hepatic vein) were also used for gas analysis and cytokine measurements. Blood gas samples were analyzed bedside (ABL 5 and Hemoximeter, Radiometer, Brønhøj, Denmark) while the other samples were centrifuged to retain plasma, which was frozen at -18° C for later analysis. Blood leukocytes and platelets were analyzed on a CELL-DYN 4000 (Abbott Laboratories, Abbott Park, IL, USA). Analyses of creatinine, alanine aminotransferase and troponin I were performed on an Architect Ci8200 analyzer (Abbott Laboratories, Abbott Park, IL, USA). Commercial porcine-specific sandwich **enzyme-linked immunosorbent assay** (ELISA) was used for the determination of TNF-α, IL-6 and IL-10 in plasma

(DY690B (TNF-α) and DY686 (IL-6), R&D Systems, Minneapolis, MN, USA and KSC0102 (IL-10), Invitrogen, Camarillo, CA, USA). The ELISAs had an intra-assay coefficient of variation (CV) of less than 5% and a total CV of less than 10%.

Ex-vivo endotoxin stimulation was performed in Paper I. 1.9 mL of arterial blood was spiked with 0.1 mL of endotoxin 200 ng x mL^{-1}, in the form of purified lipopolysaccharide from *E.coli*: 0111:B4 (Sigma Chemical Co., St Louis, MO, USA), resulting in a whole blood concentration of 10 ng endotoxin x mL^{-1}. Following incubation at 39°C for 3 h the blood samples were centrifuged to retain plasma, which was frozen at -18°C until analysis of ex vivo endotoxin-stimulated levels of TNF-α.

Urinary nitrite was measured in experiments 1 and 4. After enzymatic conversion of nitrate to nitrite by nitrate reductase, total nitrite concentration in urine was measured using the Parameter™ assay (SKGE001, R&D Systems, Minneapolis, MN, USA).

Pseudomonas aeruginosa

Bacterial inocula. The aim of the bacterial preparation was to produce a 20 mL bolus of 10^{11} **colony forming units** (cfu) of *P. aeruginosa*. The strain was isolated from a previous porcine experiment and naturally resistant to cefuroxime. It was O-antigen serotyped to O3 by a slide agglutination test with commercial antisera (Bio-Rad Laboratories AB, Solna, Sweden) at the section for Clinical Microbiology and Infectious Medicine (Uppsala, Sweden). Bacteria from over night cultures on Cystine-Lactose-Electrolyte-Deficient (CLED) agar (BD Diagnostics, Stockholm, Sweden) were dissolved in lysogeny broth (LB) according to Miller (VWR, Leuven, Belgium). The **optical density** of the bacterial solution was measured with light absorbance spectrophotometry at a wavelength of 595 nm; a target value of 0.7 was reached by either dilution of the suspension or addition of more bacteria. One hundred mL of the final suspension were further diluted with another 100 mL of LB and incubated at 37° C for 60 min. The incubated solution was centrifuged for 10 min at 20° C to form bacterial pellets that were dissolved in 20 mL of sodium chloride 0.9%. One hundred µL were diluted $1{:}10^{7}$ to confirm the concentration of the bolus dose by culture on CLED agar.

Bacterial cultures and lung weight measurements. Lung tissue bacterial cultures and weight measurements were based on three dorsal samples from the right lung cranial, middle and caudal lobes, as well as three corresponding level samples from the left lung. The six samples, ranging from 10-40 grams (g), from each animal were weighed directly and after drying for 12 h at 60^{o} C. Before drying, approximately 1 g from each sample was used for bacterial cultures. Three mL of sodium chloride 0.9% were added, fol-

lowed by 4 min of mechanical homogenization with a Stomacher 80 Biomaster (Seward, Worthing, UK). One hundred µL were sequentially diluted until $1:10^4$ and cultured in a single repetition on CLED agar plates over night at 37^{o} C. The number of cfu from the countable plates was converted to cfu x g^{-1} lung tissue.

Statistical analyses

All experiments dealt with simple variable- and repeated measure variable group comparisons. Experiments I and IV compared three groups. To reduce the amount of statistical analyses ***post-hoc*** tests were performed between individual groups only if the primary test between all three groups was significant. Experiments II and III compared two groups. Statistica™ (Statsoft, Tulsa, OK) was used in the statistical calculations and for the control of relevant assumptions. A p-value of <0.05 was considered significant in all experiments.

(I, II). The primary endpoint variables TNF-α, IL-6 and IL-10 concentrations were log-normally distributed and therefore logarithmically transformed for the statistical analyses. Baseline differences for variables approximating a normal distribution were analyzed with **multiple analysis of variance** (MANOVA), whereas non-normally distributed variables were analyzed with **Kruskall-Wallis´** test. The main statistical analysis for the primary endpoint variables was **MANOVA for repeated measures**, analyzing the group effect between all three groups during the entire experimental period and not at individual time points. The same strategy was applied to the normally distributed secondary outcome variables. Secondary outcome variables with a normal distribution were indexed to reduce the effect of inter-animal variation. Variables that were non-normally distributed were analyzed for group differences with **Mann-Whitney U test** for each time point from 0 to 5 h.

(III, IV). The same primary statistical model was used in experiments III and IV, where the major outcome variables were based on lung tissue samples. These six samples from each animal, taken at the same time were internally dependent. To keep to one main statistical method, the repeated measure variables (previously analyzed with repeated measures ANOVA in I and II) were analyzed with the same method as the lung tissue samples. A **general linear model** was used for group comparisons in the lung tissue sample variables (i.e. bacterial growth and wet-to-dry ratio) and in the repeated-measure variables. Random effects were introduced into the model to account for the within- subject dependencies of the lung tissue samples and the repeated measures respectively. Because the bacterial inocula were delivered blindly to either the right or left lung, the tissue samples in each animal were

statistically analyzed using three levels (cranial, middle and caudal) each consisting of the right and left corresponding samples.

Cytokines in arterial plasma, inoculation dose and bacterial growth in lung tissue approximated lognormal distribution and were logarithmically transformed before analysis. All bronchoalveolar lavage (BAL) variables were of non-normal distribution and thus groups were compared with **Mann-Whitney U tests**. For data symmetry, the BAL cytokines were logarithmically transformed in the table presentation. In experiment IV, comparative group statistics were based on data from the last six h of the experiment. Inoculated dose and bacterial counts in BAL were analyzed with **Kruskall-Wallis´ test**.

Protocols

Paper I. An overview of the experimental design is given below (*Figure 5*). An infusion of endotoxin, *E.coli*: 0111:B4 (Sigma Chemical Co., St Louis, MO, USA), was started at 0.25 μg x kg^{-1} x h^{-1} at 0 h. The animals were randomized in blocks of 10 to either of three groups: Prot-7h, i.e. protective ventilation for 7 h (n=10), Prot-5h, i.e. protective ventilation for 5 h (n=10) and Control, i.e. a control group (n=10). The animals in the Prot-7h group were ventilated with low V_T, 6 mL x kg^{-1}, for the entire experiment. The Prot-5h group was ventilated with a V_T of 10 mL x kg^{-1} during experimental surgery, i.e. between -2 h to 0 h, after which V_T was adjusted to 6 mL x kg^{-1} for the remaining 5 h. The animals in group Control was ventilated with a V_T of 10 mL x kg^{-1} during the entire experiment. All groups were ventilated with a PEEP of 5 cm H_20 between -2 h and 0 h. After baseline and during the remaining 5 h of the experiment, groups Prot-7h and Prot-5h were ventilated with a PEEP of 10 cm H_20, whereas the control group continued with a PEEP of 5 cm H_20. The initial respiratory rate was 25 x min^{-1} for the groups with V_T 10mL x kg^{-1} and 35 for the group with V_T 6mL x kg^{-1}. At -2 h, 0 h and thereafter hourly the respiration was adjusted to result in arterial tension of carbon dioxide ($PaCO_2$) between 5.0 - 5.5 kPa. The mode of ventilation was volume controlled with an inspiratory:expiratory (I:E) ratio of 1:2. At 1 h, cefuroxime 20 mg x kg^{-1} was given as a slow injection to prevent bacterial contamination of the model.

Interventions. Inspired oxygen fraction (FiO_2) was initially 0.3. Adjustments were made in 0.1 increments of FiO_2 at an arterial oxygen tension (PaO_2) <12 kPa and decrements of 0.05 at PaO_2>18 kPa. $PaCO_2$ was kept at values between 5.0 and 5.5 kPa by adjusting respiratory rate by increments/decrements of 10%. Within 90 minutes from the start of the endotoxin infusion, epinephrine 0.1 mg was given if mean arterial pressure (MAP) approximated mean pulmonary arterial pressure (MPAP). If MAP equaled MPAP after 90 minutes, norepinephrine infusion 20 μg x mL^{-1} was started with 1 mL bolus and an initial rate of 5 mL x h^{-1}. The procedure was repeated with doubling of the infusion rate if MAP relapsed to equaling MPAP. After 90 minutes from the start of the endotoxin infusion, isolated MAP values <50 mmHg were treated with a bolus of Ringer´s Acetate of 10 mL x kg^{-1}, maximum 15 mL x kg^{-1} x h^{-1}.

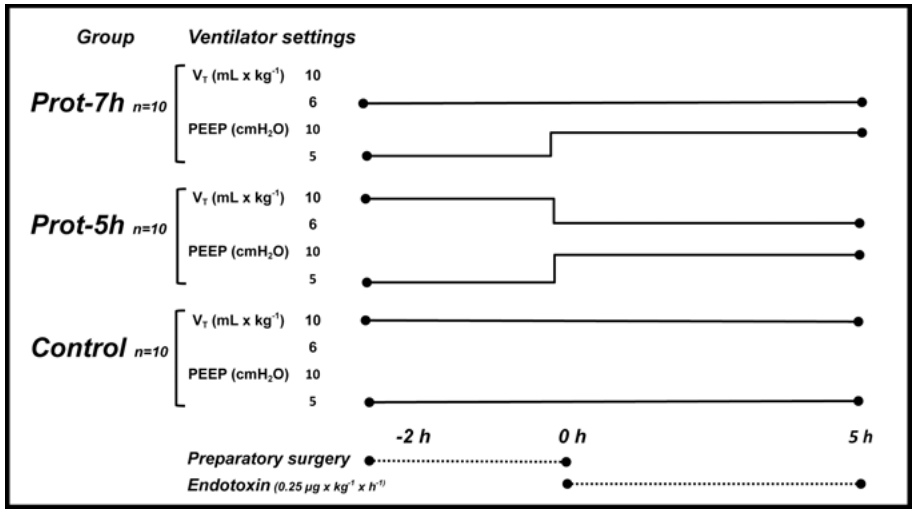

Figure 5. **Overview of the experimental design in Paper I**. All groups are n=10. Group Prot-7h was ventilated with low V_T 6 mL x kg^{-1} for the entire experiment. Group Prot-5h was ventilated with medium high V_T 10 mL x kg^{-1} for 2 h and low V_T 6 mL x kg^{-1} during the last 5 h of the experiment. Group Control was ventilated with medium high V_T 10 mL x kg^{-1} for the entire experiment. PEEP was 5 cmH_2O for all groups during the first 2 h and in the control group for the whole experiment. In groups Prot-7h and Prot-5h PEEP was 10 cmH_2O for the last 5 h. Following preparatory surgery for 2 h, an intravenous endotoxin infusion of 0.25 µg x kg^{-1} x h^{-1} was maintained for the rest of the experiment.

Paper II is based on the same experimental animals used in Paper I, but the grouping is different from 0 h. An overview of the experimental design is given below (*Figure 6*). After completion of the experimental series, differences in levels of inflammatory cytokines at 0 h between the two protective ventilation groups were compared. Given that no trend towards a difference was noted between the two groups, they were combined (coded as Prot-V, n = 20) for analysis.

Interventions. The same as for experiment I.

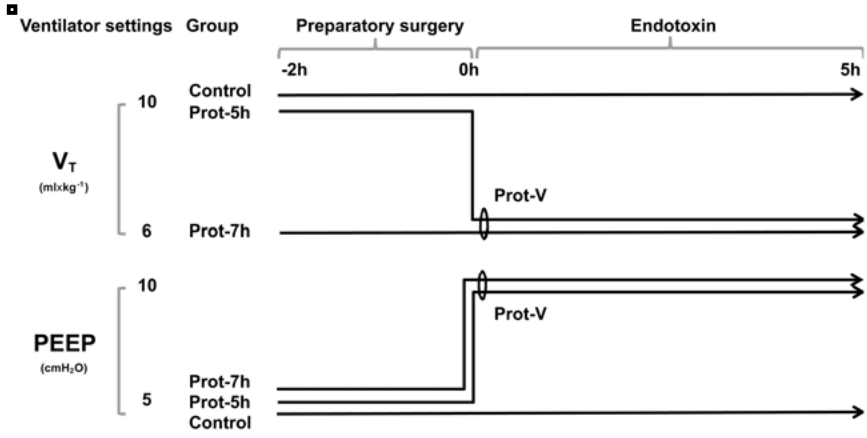

Figure 6. **Overview of the experimental design in Paper II**. During the preparatory surgery (i.e. incisions for tracheostomy and for catheters in the neck and abdomen including temporary evisceration of the spleen), all three groups are n = 10. Prot-7 h was ventilated with V_T 6 mL × kg^{-1} and PEEP 5 cmH_2O and Prot-5 h with V_T 10 mL × kg^{-1} and PEEP 5 cmH_2O from −2 to 0 h. From 0 h, Prot-7 h and Prot-5 h were combined into one group, Prot-V (n = 20), and ventilated with V_T 6 mL × kg^{-1} and PEEP 10 cmH_2O until the end of the experiment. The control group was ventilated with V_T 10 mL × kg^{-1} and PEEP 5 cmH_2O for the entire experiment. From 0 h, an endotoxin infusion was given at a rate of 0.25 µg × kg^{-1} × h^{-1} until the end of the experiment.

Paper III. An overview of the experimental design is given below (*Figure 7*). The initial respirator settings were V_T 6 mL x kg^{-1}, PEEP 10 cmH_2O, respiratory rate 35 breaths x min^{-1} in the protective group (n=10) and V_T 10 mL x kg^{-1}, PEEP 5 cmH_2O and 25 breaths x min^{-1} in the control group (n=10). Initial inspired fraction of oxygen was 0.3 for all animals. Two animals in each group served as sham animals and were not challenged with bacteria. At the start of the experimental protocol at 0 h 10^{11} colony forming units of *P. aeruginosa* were given intratracheally.

Interventions. Adjustments were made every hour to reach normal ventilation, defined as $PaCO_2$ between 4.5 and 6.5 kPa by increments or decrements of 10% of respiratory rate. PaO_2 was kept between 10 and 18 kPa by either an increment of 10% or a decrement of 5% of FiO_2. If MAP equaled MPAP within the first 90 min of the experiment, norepinephrine was given in boluses of 40 µg. If MAP equaled MPAP after 90 min, Ringer´s acetate was given in bolus doses of 15 mL x kg^{-1} maximum twice, and a norepinephrine bolus of 20 µg was given followed by a norepinephrine infusion of 20 µg x mL^{-1} with a starting rate of 5 mL x h^{-1}. If MAP regardless of MPAP were lower than 60 mmHg after 90 min or if cardiac index (CI) were below 2, a norepinephrine infusion of 20 µg x mL^{-1} was started with an initial rate of 5 mL x h^{-1} without a preceding bolus. The infusion dose was doubled every time MAP or CI relapsed under the preset limits.

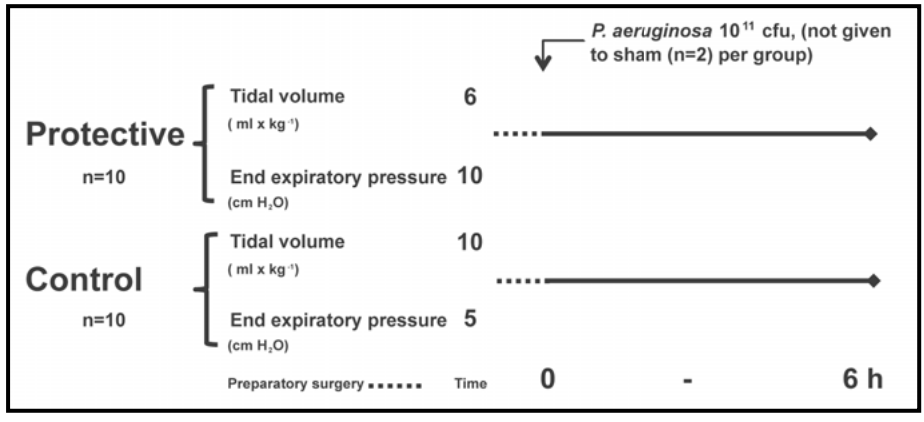

Figure 7. **Overview of the experimental design in Paper III.** Ventilator settings during the whole experiment, tracheostomy and surgical preparations for approximately one hour (dashed line) precede the inoculation of *P. aeruginosa* 10^{11} colony forming units (cfu) at the start of the experimental protocol at 0 h.

Paper IV. An overview of the experimental design is given below. Two groups, VAP 30 + Etx (n=6) and VAP 30 (n=6) were studied over 30 hours. The third group VAP 6 (n=8) was derived from Paper III (i.e. group Control without sham). The *P. aeruginosa* pneumonia phase made up the last 6 hours in all three experiment groups. During the first 24 h in groups VAP 30 + Etx and VAP 30, the pigs were ventilated in lateral position with 180◦ position changes every 6 hours. During the last 6 hours, the animals from all groups were in the supine position. Group VAP 30 + Etx received an endotoxin infusion of 0.063 µg x kg^{-1} x h^{-1} for 24 hours (*E.coli*: 0111:B4 (Sigma Chemical Co., St Louis, MO, USA)), and VAP 30 received an equivalent amount of saline 0.9%. After 24 hours, an intratracheal inoculum of 10^{11} colony forming units of *P. aeruginosa* type O3 was given. Group VAP 6 received no pre-exposure to ventilation or endotoxin but started the experimental protocol from the bacterial inoculation.

Interventions. Initially, V_T was 10 ml x kg^{-1}, PEEP 5 cm H_2O, respiratory rate 25 x min^{-1} and FiO_2 0.3. The respiration was adjusted to meet a $PaCO_2$ value between 4.5 and 6.5 kPa by increments or decrements in respiratory frequency of 10%. The maximum frequency to achieve this goal was 50 breaths per minute. Predefined increments of FiO_2, 0.3-0.6-0.8-1.0, were performed at PaO_2 values below 10 kPa simultaneously with changes in predefined PEEP levels of 5-8-10-14 cm H_2O. If the resulting plateau pressure was over 30 cm H_2O, V_T was reduced to 7 mL x kg^{-1} and the I:E ratio was changed from 1:2 to 1:1.

Alveolar recruitment maneuver (ARM) consisted of stepwise increments of PEEP until peak pressure was 35 cm H_2O, followed by prolonged inspiration for 10 s. ARM was performed at the start of the protocol (-24 h) and after each position change in the 30 h groups, and after bacterial inoculation at 0 h

in all groups. During the first 90 min of the experiment, norepinephrine was used in boluses of 40 µg if mean arterial pressure (MAP) equaled mean pulmonary arterial pressure (MPAP). A MAP value, regardless of MPAP, below 60 mmHg after 90 min was treated with a bolus of Ringer´s acetate 15 mL x kg^{-1}, a 1 mL bolus of norepinephrine 20 µg x mL^{-1} followed by a norepinephrine infusion of the same concentration starting at 5 mL x h^{-1}. At a relapse of MAP below 60 mmHg, the infusion dose was doubled.

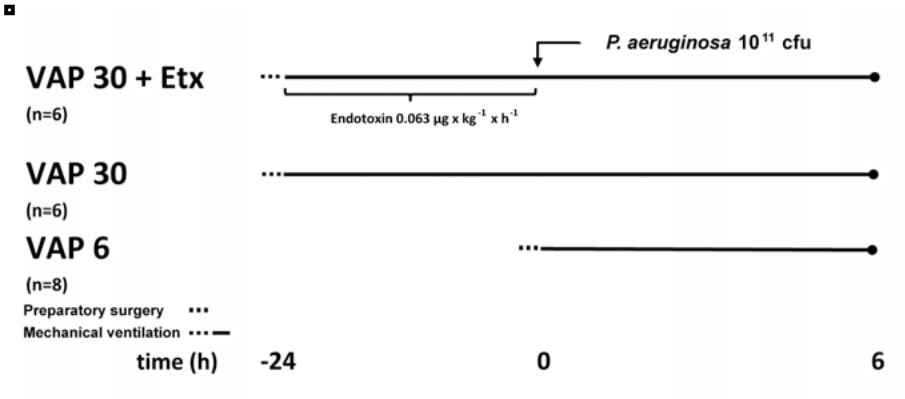

Figure 8. **Overview of the experimental design in Paper IV**. Solid black lines denote mechanical ventilation, dashed black lines denote the preparatory surgery. The arrow indicates the start of the pneumonia phase with an inoculation of 10^{11} colony forming units of *P. aeruginosa*.

Results, Discussions and Conclusions

The following accounts will summarize for each experiment the main points in the results, the discussion relevant to them and the conclusions. The full accounts can be found in the Papers I-IV. For readability, tables are omitted and referred to the original document when necessary.

A total 83 animals were used in all papers, 63 were included in the results. Papers **I** and **II** used the same animals. Out of a total of 42 animals 30 were included. Of the 12 animals that were not included 5 were used for pilot experiments and 7 were excluded because of early death. All were replaced according to protocol. Papers **III** and **IV** used a total of 41 animals out of which 33 were included. All of the 8 non-included animals were used for pilot experiments, hence no animal died during the final experiments. 8 animals from Paper III were used as a control group in Paper IV.

Paper I

Results.

Inflammatory responses. No differences between groups were noted for the TNF-α values, but there were significant differences between groups in IL6 and in IL10. *Post hoc* analysis demonstrated lower IL6 values in the Prot-7h group than in the control group ($p<0.05$), with the most marked differences at the end of the experiment. IL10 values were, in the *post hoc* analyses, lower in the Prot-7h ($p<0.05$) and Prot-5h ($p<0.05$) groups than in controls, with the most marked differences at the end of the experiment (*Figure 9*).

The propensity to produce TNF-α after ***ex vivo*** **endotoxin stimulation** at 0 h, ΔTNF-α, was lower in the Prot-7h group, where 8/10 animals showed a completely suppressed cytokine production *ex vivo* compared with the Prot-5h ($p<0.05$) and control groups ($p<0.05$) (*Figure 10*). At 2 h, complete suppression of ΔTNF-α in the Prot-7h group was no longer present and differences between the groups were no longer apparent (data not shown).

Urinary total nitrite was higher at 2 and 4 h in the Prot-7h group than in both the control group ($p<0.05$ and $p<0.05$ respectively) and the Prot-5h group ($p<0.05$ and $p<0.05$ respectively) (*Figure 11*).

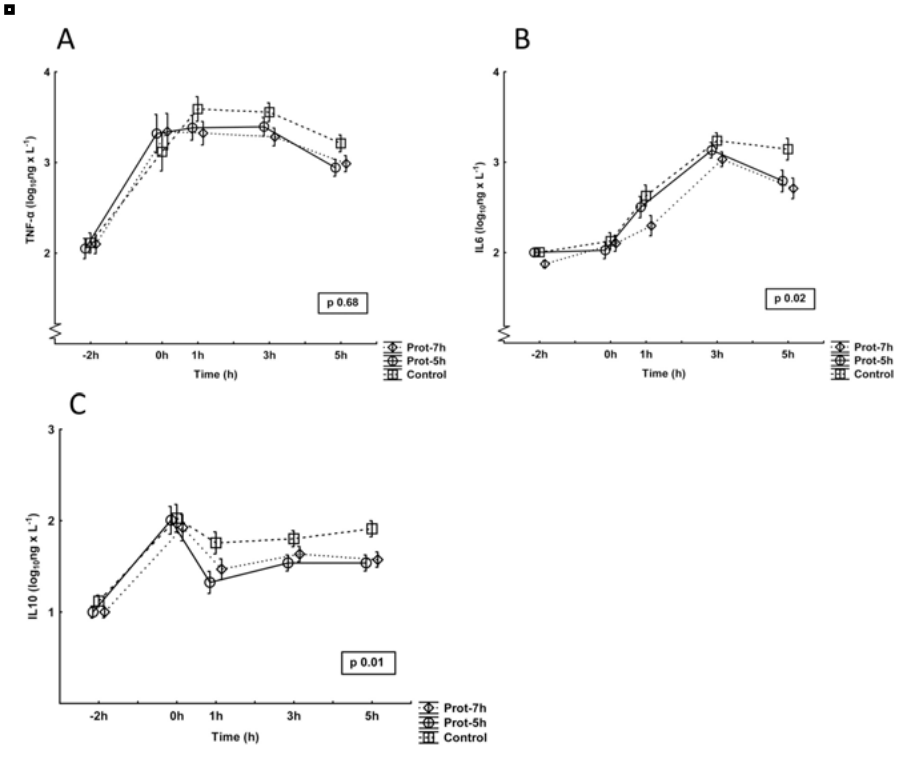

Figure 9. Arterial plasma levels of A) tumor necrosis factor α (TNF-α), B) interleukin 6 (IL6), C) interleukin 10 (IL10) during the experiment. All groups are n=10. The values have been logarithmically transformed. Mean±SE. The p-value is the result of a multiple ANOVA (MANOVA) for repeated measures comparing differences between all three groups during the entire experiment period.

Physiology and organ dysfunction. In the respiratory variables PaO_2/FiO_2 displayed significant differences between groups. *Post hoc* analyses showed that the Prot-7h group had higher values than the control group during the experiment ($p<0.05$) whereas only a trend toward significance compared to the Prot-5h group ($p=0.07$) was seen. Significant differences between groups were seen in circulatory variables such as cardiac index, oxygen delivery index (DO_2I) and left ventricular stroke work index (LVSWI). *Post hoc* analyses revealed that the Prot-7h group decreased more in CI, most evident at 5 h, than the control group ($p<0.01$). A highly significant reduction in DO_2I was found in the Prot-7h group as compared with the control group ($p<0.01$). In LVSWI both the Prot-7h and Prot-5h groups showed significantly more reduced values than the control group ($p<0.05$ and $p<0.01$). (*Table 3* in the original document). There were no differences in pH, arterial lactate, portal lactate, creatinine or alanine amino transferase between the groups. The animals in the control group increased more in troponin I at the end of the experiment compared with the animals in the Prot-5h group

(p<0.05). Between the control group and the Prot-7h group only a trend towards a difference was seen (p=0.08) (*Table 4* in the original document).

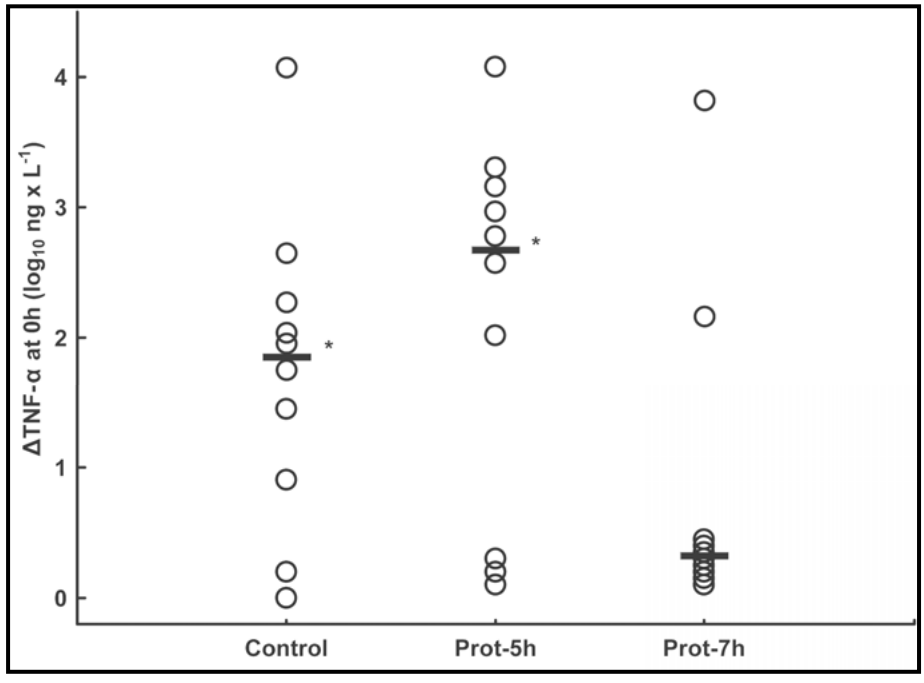

Figure 10. Endotoxin-induced whole blood tumor necrosis factor α production ex vivo, Δ-TNF-α, in the immediate postoperative period at 0 h. All groups are n=10. The values are given on a logarithmic scale as a scatterplot. The horizontal bar denotes the median. The * denotes a significant difference compared with the Prot-7h group, p<0.05, Mann-Whitney U test.

Discussion

Lower plasma levels of IL6 and IL10 indicated the attenuation of the inflammatory response in the protectively ventilated animals. Additionally, in the animals that were protectively ventilated through the whole experiment the ex-vivo endotoxin stimulation of blood showed a picture of immunotolerance, and the urinary nitrite levels were higher.

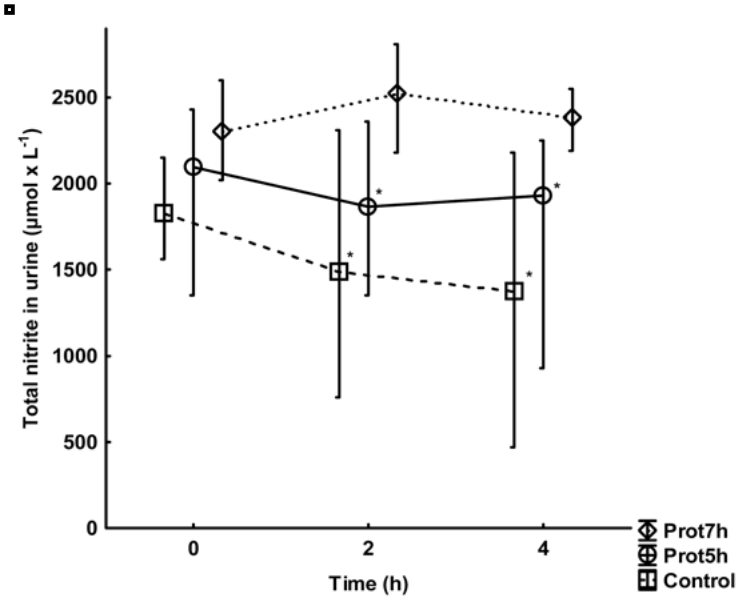

Figure 11. Total nitrite concentration in urine. All groups are n=10. Values are median and interquartile range. The * denotes a significant difference compared with the Prot-7h group, $p<0.05$, Mann-Whitney U test.

The induction of endotoxin tolerance or immunotolerance is highly complex as recently modeled by Fu et al. [136]. Endotoxin tolerance has been observed in patients with sepsis, trauma, pancreatitis and in patients surviving cardiac arrest [137]. Moreover, it has been associated with the level of disease in ARDS [138]. The role of inducible nitric oxide synthase (iNOS) in the induction of endotoxin tolerance was elegantly shown by Dias et al. in a murine model. Endotoxin tolerant animals were given the specific iNOS antagonist aminoguanidine returned to the native response to endotoxin in a similar manner to iNOS knockout mice [139]. Vobruba et al. published higher nitrite/nitrate and iNOS levels in bronchoalveolar lavage from pigs ventilated with a V_T of 7 mL x kg^{-1} compared with animals ventilated with a V_T of 15 mL x kg^{-1} [140]. In the current study, the increased nitrite levels in urine in the protectively ventilated animals support the results of Vobruba et al. and serve as a mechanistic explanation to induction of endotoxin tolerance following protective ventilation. It has been questioned whether the compensatory rise in respiratory rate following low V_T ventilation can lead to injurious effects [141]. Higher respiratory rate in spontaneously breathing rats led to increased activation of lung tissue metalloproteinases and reduced integrity of the extracellular matrix in the lung [142]. Wang et al. reported an association between metalloproteinase 9 and upregulation of iNOS via nuclear factor-κβ [143]. It might be hypothesized that the higher respiratory

rate in low V_T ventilation leads to effects on the lung extracellular matrix, which in turn induces iNOS and endotoxin tolerance.

The lung protective ventilation led to less pulmonary dysfunction and better-preserved FRC after 5 h of endotoxemia. The concept of lung biotrauma and subsequent non- pulmonary organ injury has attracted scientific interest as a model of multiple organ failure [52,144,145]. The animals ventilated with a low V_T increased less in plasma troponin I than the animals in the control group, indicating a less prominent endotoxin-induced cardiac injury following preventive lung protective ventilation. These beneficial results of low V_T ventilation on non-pulmonary organ injury are especially noteworthy considering that they were observed despite decreased macro-circulatory variables, e.g. reduced CI and DO_2I. The greater decrease in macro-circulatory variables seen in the protectively ventilated animals is probably explained by the higher PEEP, leading to higher intrathoracic pressure and subsequent lower venous return [146].

What the present study adds is the beneficial effect of preventive treatment with low V_T ventilation 2 h before an experimental septic insult. The inflammatory response to standardized surgery together with mechanical ventilation did not differ between the groups at 0 h. Similarly, the pulmonary function and the other organ function variables were the same in all groups at 0 h. Despite this observation, there were obvious inflammatory differences between the groups induced by the simulated postoperative complication, i.e. the endotoxin infusion, which indicates that one main effect of different perioperative ventilator protocols may be induction of immunotolerance after low V_T ventilation. This assumption is reinforced with the endotoxin tolerant feature of the protectively ventilated animals associated with higher urinary nitrite levels

Conclusions

Low V_T ventilation combined with higher PEEP in healthy animals exposed to surgery and experimental postoperative sepsis led to a less prominent systemic inflammatory response, pulmonary dysfunction and cardiac injury when compared with animals ventilated with medium-high VT and lower PEEP. Preventive low V_T ventilation was associated with immunotolerance and higher nitric oxide production, which could be a mechanistic explanation for the attenuation of systemic inflammation.

Paper II

Results.

Comparison between cytokine levels in different sample locations.
The levels of cytokines from the four plasma sample locations are presented in *Figure 12*. TNF-α levels were higher in the hepatic vein than in the artery, jugular bulb and portal vein. IL-6 levels were higher in the artery and jugular bulb than in the portal and hepatic veins. No difference was found between IL-6 levels in the jugular bulb and the artery, nor were there differences between the portal and hepatic veins. IL-10 levels were numerically, but not significantly, higher in the portal vein compared with the jugular bulb and hepatic vein. IL-10 levels were lower in the artery than in the portal vein, hepatic vein and jugular bulb.

Comparison between protective ventilation initiated before and during endotoxin on organ-specific cytokine levels *(Rationale for the combination of the two protective groups)***.**
Levels of TNF-α, IL-6 and IL-10 in hepatic, portal and jugular bulb veins did not differ when the values at 0 h in group Prot-7 h were compared with those in group Prot-5 h (*Table 2 in original document*). Because no individual p-value was less than 0.5, the two groups were combined in the following analyses into the Prot-V group according to the statistical plan. Additionally, to rule out late effects a repeated measure ANOVA was performed between Prot-7 h and Prot-5 h groups from 0 to 5 h. No significant differences were found.

Comparison between protective ventilation and controls on organ-specific cytokine responses.
The levels of cytokines from the different sample sites comparing the two ventilation groups (Prot-V and Control) are depicted in *Figures 13-15*. The **hepatic vein** showed significantly lower values during the whole experiment in the protective group for TNF-α and IL-10, whereas IL-6 values were numerically lower in the protective group at the end of the experiment (though not statistically significant). In the **portal vein** levels of all cytokines in the protective group were numerically lower, but only reached borderline significance for TNF-α. The **jugular bulb** showed lower numerical values in the Prot-V group for TNF-α and IL-10 but did not reach statistical significance over the whole experiment. No significant differences were seen for IL- 6. In **arterial samples** no difference was noted between the ventilation groups for TNF-α; however, the control group had higher levels of IL-6 and IL-10.

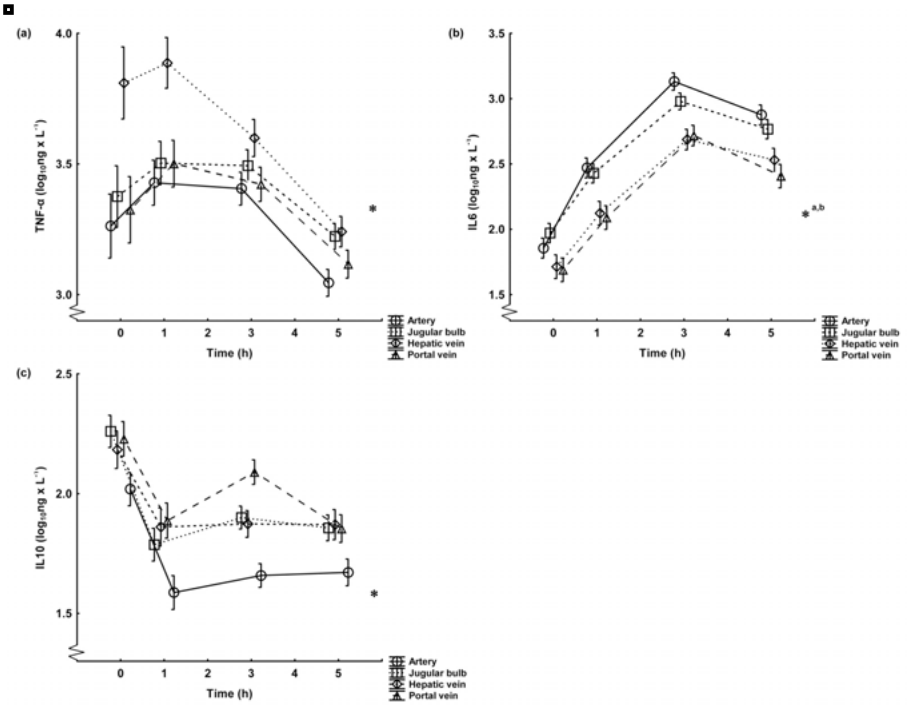

Figure 12. **TNF-α, IL-6, and IL-10 levels from different sample locations.** Values are logarithmically transformed (mean ± SD) and reflect all animals in the experiment (n = 30) from 0 to 5 hours. ANOVA for repeated measures, comparing individual sample locations over the whole experiment: a) TNF-α, *denotes the hepatic vein compared with the artery ($p < 0.001$), jugular bulb ($p < 0.001$) and portal vein ($p < 0.001$). b) IL-6, *a denotes the artery compared with the hepatic vein ($p < 0.001$) and portal vein ($p < 0.001$), *b denotes the jugular bulb compared with the hepatic vein ($p < 0.01$) and portal vein ($p < 0.01$). c) IL-10, *denotes the artery compared with the hepatic vein ($p < 0.001$), portal vein ($p < 0.001$) and jugular bulb ($p < 0.001$).

Discussion

The main findings were as follows: a) levels of cytokines differed significantly depending on sample location, b) protective ventilation initiated before endotoxin was not superior to that which was initiated concurrently with endotoxin and c) protective ventilation attenuated cytokines on an organ-specific level, most notably in the liver.

To our knowledge, no study has specifically aimed to compare cytokine levels from multiple sample sites in a large animal inflammatory model. The rational for the catheter placements in this study was based on cytokine responses previously described from these locations, i.e. the spleen and gut into the portal vein [147,148], liver [149], brain [150] and lungs [151]. Our results confirm studies that differentiate inflammatory responses from different organs and complicate the concept of 'systemic' levels of cytokines. A

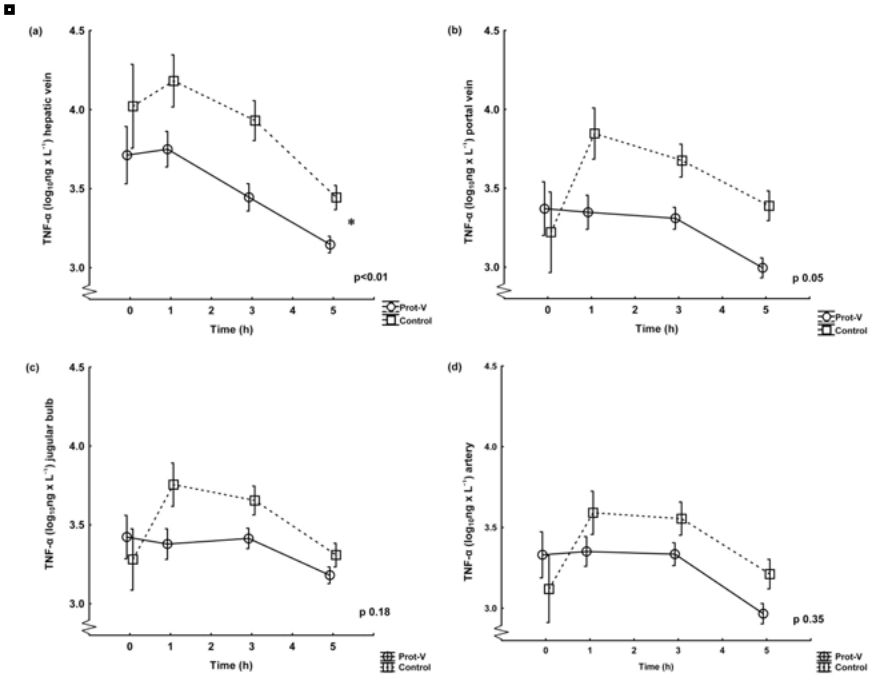

Figure 13. **TNF-α levels in the ventilation groups Prot-V and Control**. Values are logarithmically transformed (mean ± SD) from 0 to 5 hours. The Prot-V group included 20 pigs and the Control group 10. The p-values refer to ANOVA for repeated measures comparing the groups over the whole experiment. a) Hepatic vein, *denotes significance. b) Portal vein. c) Jugular bulb. d) Artery.

recent study on sepsis patients compared plasma levels of TNF-α, IL-6 and IL-10 to immunostaining of freely circulating monocytes and leukocytes. Only small amounts of these cells indicated production, which supports the hypothesis that cytokine production primarily takes place in organ-resident cells, transmigrated cells or endothelia [75]. Additionally, a study on rats compared resident macrophages from lungs, peritoneum, liver and spleen and reported differences in cytokine production after in vitro endotoxin stimulation [66]. Two theoretical scenarios serve to discuss the observed differences in cytokines levels from the different sample locations. First, regarding location; what would it look like if one organ system were dominant in cytokine production from an inflammatory stimulus? Likely, levels of all cytokines would be the highest in efferent blood from this organ in comparison with other sample locations. Second, regarding specific cytokines; what would it look like if all organ systems acted uniformly in their reaction to an inflammatory stimulus? If so, the levels of different cytokines would be stacked in the same order independently of where the samples were taken, and only the magnitude of individual cytokine levels would differ between the locations. Our results, contrarily to the two proposed

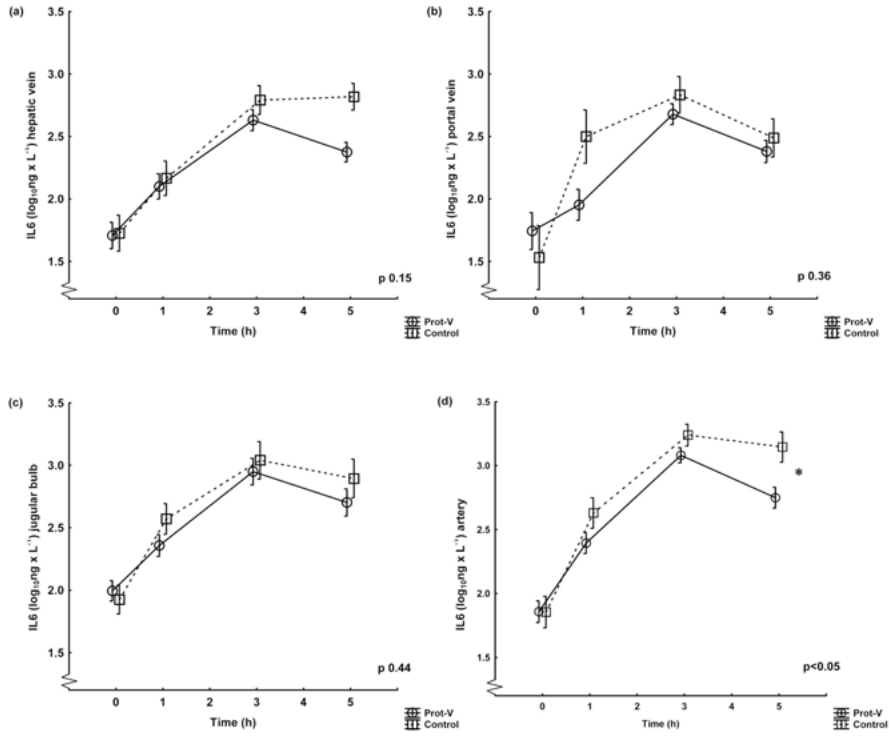

Figure 14. **IL6 levels in the ventilation groups Prot-V and Control.** Values are logarithmically transformed (mean ± SD) from 0 to 5 hours. The Prot-V group included 20 pigs and the Control group 10. The p-values refer to ANOVA for repeated measures comparing the groups throughout the experiment. a) Hepatic vein. b) Portal vein. c) Jugular bulb. d) Artery, *denotes significance.

scenarios, strongly indicate that peak levels of different cytokines are located at different locations in the body, and that different organ systems preferentially produce certain cytokines. Most clearly, the levels of arterial cytokines – comparably the lowest in TNF-α and IL10, but the highest in IL6, illustrate this conclusion. The blood–brain barrier could potentially present a hinder to differentiate arterial levels from brain-derived levels in the jugular bulb, which makes these locations especially interesting to compare. The fact that IL10 levels significantly separate these two locations indicates that jugular bulb levels are not solely products of arterial levels.

Our study did not demonstrate any significant differences in cytokine expression between the two initial protectively ventilated groups (i.e. Prot-7 h and Prot-5 h) during surgery between −2 h and 0 h. The distinct reaction to surgery seen at 0 h in TNF-α and IL-10 would reasonably differentiate between two ventilation modes that were not equal in inflammatory attenuating ability. One reason for the failure to find differences could be the lack of

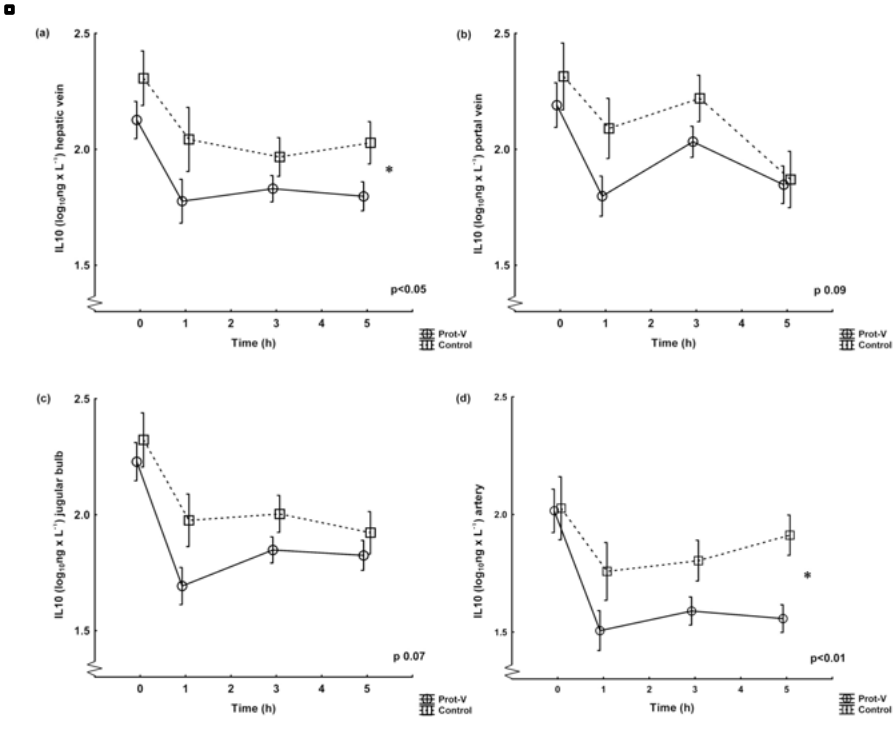

Figure 15. **IL10 levels in the ventilation groups Prot-V and Control.** Values are logarithmically transformed (mean ± SD) from 0 to 5 hours. The Prot-V group included 20 pigs and the Control group 10. The p-values refer to ANOVA for repeated measures comparing the groups over the whole experiment. a) Hepatic vein, *denotes significance. b) Portal vein. c) Jugular bulb. d) Artery, *denotes significance.

power for this particular outcome measure. Another reason could be that the groups were only separated by tidal volume and not by PEEP during surgery. Hypothetically, if PEEP were the dominant factor in our combined intervention, a difference would not be expected in such a relatively short surgery time as 2 h. The effects of protective ventilation could be seen in all the sample locations in this experiment. Although not reaching significant differences for all cytokines, all locations displayed lower absolute values in the protectively ventilated group as compared with the control group. The relatively small difference in tidal volume between the groups is unlikely to lead to such rapid and uniform repression of the inflammatory response from different organs. The adverse effects from the larger tidal volume would come from a massive alveolar overstretch mechanism, and in this regard even the control group had clinically moderate tidal volumes. More likely, the cause would be the combined intervention or the PEEP level singularly. Interestingly, our observations suggest a general attenuation of inflammation induced by small differences in PEEP and tidal volume, but with a differen-

tial impact on organs. The possible mechanistic connection between mechanical ventilation and central neurogenic suppression of systemic inflammation is a highly promising research line [152].

The clinical implication of this study stems primarily from the finding that protective ventilation affects the TNF-α levels of the hepatic efferent circulation, which have previously been correlated with hepatocellular dysfunction and severity of Acute Respiratory Distress Syndrome [153,154]. The attenuation induced by protective ventilation on hepatic TNF-α production would therefore affect these areas in a clinically beneficial way and further favor the use of protective ventilation regimes outside of the ARDS domains. The chosen animal model in our study bridges the results from experiments performed on smaller animals [28,49], largely because humans share certain physiological and anatomical similarities with pigs but not with mice, rodents and rabbits [107].

Conclusions

Cytokine output is differential between organs during experimental sepsis. We see no clinical implication from cytokine levels in this model for initiating protective ventilation before endotoxemia. However, during endotoxemia protective ventilation will attenuate hepatic inflammatory cytokine output and consequently reduce the total proinflammatory burden.

Paper III

Results

Bacterial cultures and bronchoalveolar lavages

P. aeruginosa growth in lung tissue (cfu x g^{-1}) was lower in the protective group than in the control group. Both groups displayed the highest bacterial counts at the caudal level and the lowest at the cranial level (*Figure 16*). Bacterial growth in BAL at 0 and 6 h did not differ between the groups. Both TNFα and IL6 levels in BAL were lower in the protective group at the start of the experimental protocol at 0 h, but the differences did not persist at the end of the experiment (6 h) (*Table 1 in original document*).

Plasma cytokines, inflammatory cells and nitrite

No differences were detected between the groups in arterial plasma levels of TNFα or IL6, whereas IL10 was lower in the protective group. Platelet counts were lower in the protective group, but there were no group differences in leukocytes, neutrophils, or total nitrite levels in urine (*Table 2 in original document*).

Lung injury, physiologic variables and hypoperfusion

PaO_2/FiO_2 was higher in the protective group than in the control group (*Figure 17*). In contrast, the wet-to-dry ratio was lower in the protective group (*Figure 18*). Peak airway pressure did not differ between the groups, whereas mean airway pressure was higher in the protective group and plateau pressure was higher in the control group. CI, MAP and MPAP were lower in the protective group, but heart rate and pulmonary capillary wedge pressure displayed no group differences. Temperature was lower in the protective group. There were no group differences in lactate levels in arterial or portal blood (*Table 3 in original document*).

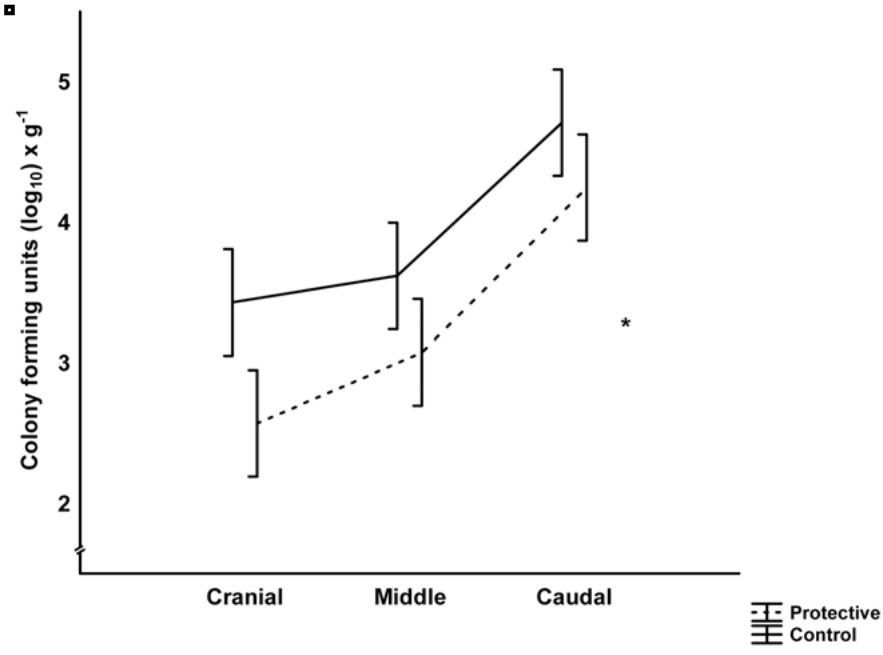

Figure 16. ***P. aeruginosa*** **growth in lung tissue**. *P. aeruginosa* counts at the three sample levels (cranial, middle, caudal), log_{10} colony forming units per gram lung tissue, mean±SEM, * denotes $p<0.05$ between the protective and control groups from the general linear model analysis.

Discussion

We have shown that protective ventilation, with medium low V_T and medium high PEEP can affect the course of experimental pneumonia compared with a control group. Specifically, *P. aeruginosa* counts in lung tissue were significantly reduced and the development of lung injury was attenuated over a 6-h experiment.

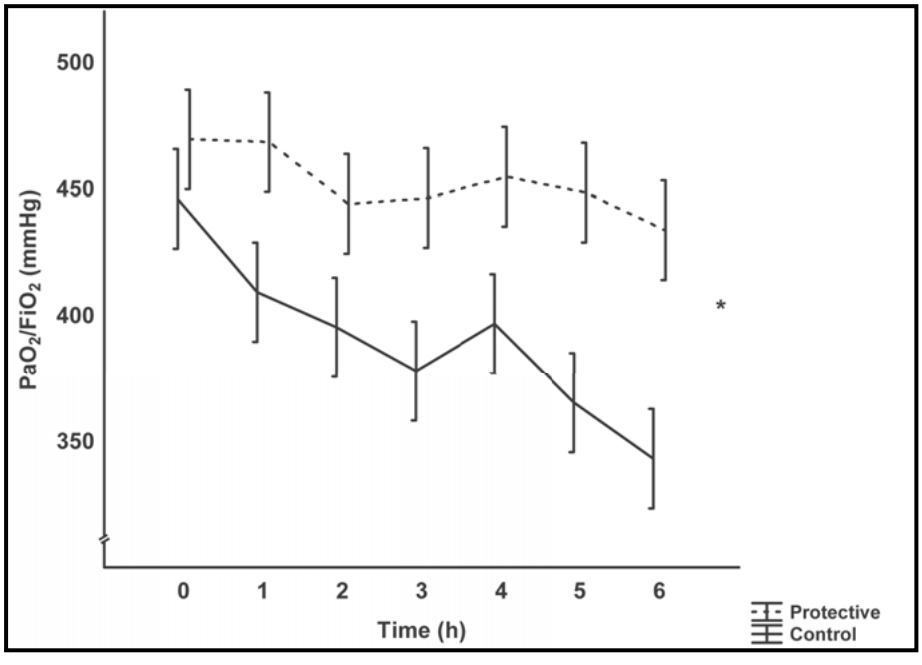

Figure 17. **PaO_2/FiO_2 (mmHg).** Arterial tension of oxygen / inspired oxygen fraction from 0 to 6 hours, mean±SEM, * denotes $p<0.01$ between the protective and control groups from the general linear model analysis.

The bacterial count in lung tissue is a clinically relevant outcome measure. The magnitude of the bacterial reduction in lung tissue from protective ventilation in the current experiment was similar to that seen in a porcine model with *P. aeruginosa* pneumonia over 72 h, comparing treatments with or without antibiotics [155]. Hypothetically, how could our protective ventilation counteract bacteria in healthy lungs that were exposed to a massive load of *P. aeruginosa*? The answer may lie in bacterial inhalation studies in mice showing that the phagocytic action of alveolar macrophages (AM) is the major mechanism of early resistance to bacterial infection [156]. The phagocytic action from AM is rapid, leaving few bacteria free to multiply after only a few hours [157]. Still, both phagocytosis and bacterial killing can be affected by ventilation with or without the presence of inflammation. Early pneumonia experiments on dogs demonstrated improved AM phagocytic action from the addition of PEEP alone [158]. Endotoxin-induced systemic inflammation and lung injury models affect both AM and interstitial macrophages of the lung to display defect phagocytosis and bacterial killing [159,160]. PEEP can prevent dispersion of alveolar fluid to different parts of the lungs in rats, which in turn could mediate stabilization of the infection locally [161]. The reduction of atelectasis with PEEP has previously been correlated with greater bacterial clearance in pigs [162]. In the setting of ALI

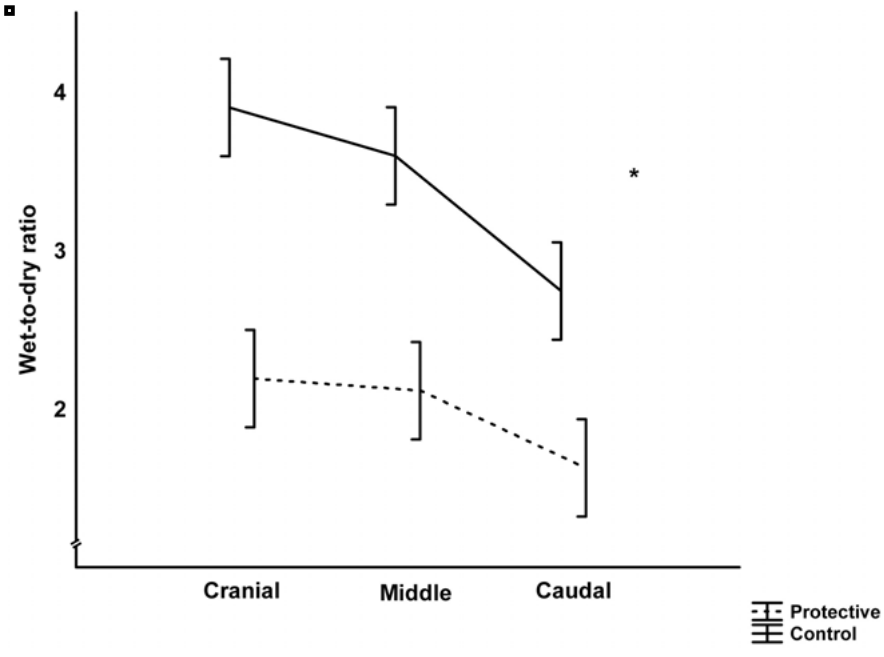

Figure 18. **Wet-to-dry ratio.** Wet weight / dry weight of lung tissue at the three sample levels (cranial, middle, caudal), mean±SE, * denotes $p<0.01$ between the protective and control groups from the general linear model analysis.

the reduction of atelectasis using an open lung approach with PEEP and surfactant has shown lower bacterial counts in lung tissue and blood [163]. However, PEEP levels that raise plateau pressures and overstretch alveoli at end-inspiration will probably counteract any protective effect [164]. Edema formation in rat lungs has been correlated to bacterial viability and to levels of clearance in vivo, and to impaired bactericidal activity of AM in vitro [165]. The local pro-inflammatory environment in the lung alveoli is affected by ventilation. Ventilator settings that permit either cyclic atelectasis or excessive stretch of alveoli will produce TNFα in BAL [166,167]. TNFα in BAL has been linked to endothelial damage and edema formation in early stages of experimental ARDS [168]. In a rodent experiment with *P. aeruginosa* inocula and lung separation, low V_T ventilation yielded less edema and lower TNFα formation in the non-involved lung segments [169]. The avoidance of adverse ventilator settings is a probable cause for the lower bacterial counts in lung tissue, less lung damage and reduced bacterial decompartmentalization seen in dog experiments [53,170]. The protective group had lower plateau and higher mean airway pressures which potentially counteracted atelectasis and excessive stretch of alveoli to a greater extent than in the control group. Additionally, the fixed PEEP levels resulted in reduced driving pressure (plateau minus PEEP) in the protective group, which constitutes a factor previously linked to favorable outcome in clinical

studies [171]. Bacterial growth in lung tissue differed between the protective and control groups; however, bacterial growth, TNFα and IL6 in BAL did not differ between the groups at the end of the experiment. The disparate results might be attributable to several factors. Regions of atelectasis are thought to have reduced bacterial clearance [162,172]. The lung damage variables (PaO_2/FiO_2 and wet-to-dry ratio) show that the control group deteriorated toward ARDS and had more lung water than the protective group. Reasonably, at 6 h, the airways were relatively more open in the protective group. Hypothetically, the BAL fluid in the control group may not have sampled far enough peripherally where bacterial or cytokine counts differed because of airway closure. We have previously noted peak values for TNFα and IL6 in plasma in inflammatory models at 3 h after insult [173], and we cannot rule out differences in in the current experiment between 0-6 h. Moreover, given the unspecific nature of our BAL procedure, it is possible that the measurement was underpowered. The lower TNFα and IL6 counts in BAL in the protective group at 0 h are noteworthy as the two groups had not yet deteriorated in lung function. The relevance of reducing the transient pro-inflammatory TNFα peak seems especially important in early stages of lung injury to prevent deterioration [167].

The main benefit and translational relevance of the current study is that the large animal model resembles the clinical respiratory situation for intensive care-treated pneumonia patients in early phase. Furthermore, the ventilator settings were common to everyday clinical practice.

Conclusions

In early stage pneumonia in healthy lungs protective ventilation with medium low V_T and medium high PEEP has the potential to significantly reduce both pulmonary bacterial burden and the development of lung injury. Hypothetically, higher PEEP kept the alveoli open and available to local immune cells while the low V_T reduced stretch-induced local inflammation in the face of increasingly dishomogeneous lungs.

Paper IV

Results

***P. aeruginosa* in lung tissue and bronchoalveolar lavage**

There were no differences in inoculation doses of *P. aeruginosa* between the groups (*Table 1 in original document*). The lowest bacterial counts in lung tissue were in group VAP 6, and the highest in VAP 30 + Etx. There were significant differences between all groups (*Figure 19*). Bacterial counts in BAL showed no group differences (*Table 1 in original document*).

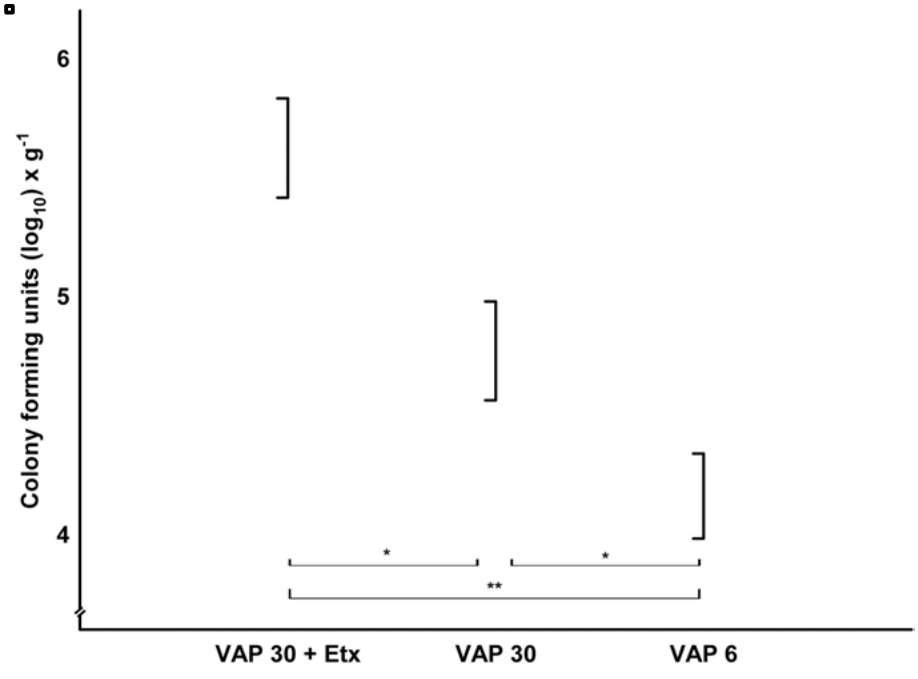

Figure 19. **Bacterial growth in lung tissue**. *P. aeruginosa* counts, log_{10} colony forming units per gram lung tissue, mean±SEM, general linear model analysis, * denotes $p<0.05$, ** denotes $p<0.01$.

Physiologic, lung damage and hypoperfusion variables

In the respiratory variables group VAP 30 + Etx had higher levels of peak, plateau and mean airway pressures, and of respiratory rate than both the other groups. The VAP 6 group had higher tidal volume than both the other groups. PaO_2/FiO_2 was higher in group VAP 6 than in both the other groups (*Figure 20*). Wet to dry weight ratio was lowest in group VAP 30, highest in VAP 30 + Etx with VAP 6 in an intermediate position (*Figure 21*). In the circulatory variables group VAP 6 had lower heart rate, cardiac index and mean arterial pressure, and higher mean pulmonary arterial pressure and pulmonary capillary wedge pressure than both the other groups. (*Table 2 in original document*).

Inflammatory variables

Plasma levels of TNFα (*Figure 22*) and IL10 were higher in VAP 6 than in both the other groups, but there were no group differences regarding IL6. There were no group differences in BAL TNFα or IL6. Platelets and urinary

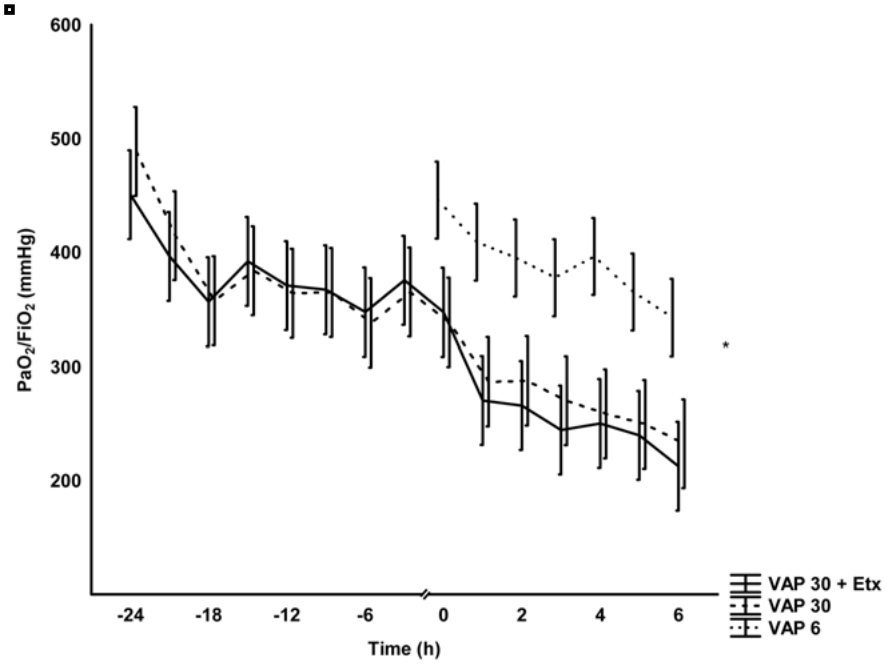

Figure 20. **PaO_2/FiO_2 (mmHg)**. Arterial tension of oxygen / inspired oxygen fraction (PaO_2/FiO_2), mean±SEM, general linear model analysis based on data from 0 to 6 h between all groups, * denotes $p<0.001$, axis scale changes at 0 hours.

nitrite were highest in group VAP 6. Leukocytes were highest in group VAP30 + Etx, but there were no group differences regarding neutrophils. The temperature was higher in VAP 30 than in both the other groups (*Table 3 in original document*).

Discussion

Exposure to mechanical ventilation for 24 hours before a bacterial challenge showed increased *P.aeruginosa* counts in lung tissue after 6 h compared to animals that were not exposed before the challenge. The addition of systemic inflammation during the 24 h further enhanced the *P.aeruginosa* counts.

In this study we see clear differences between the groups in inflammatory variables. TNFα and IL10 levels in plasma were highest in the 6-hour group, but IL6 did not differ at group level. Endotoxin tolerance, or immunotolerance, can be described as a blunted reaction to a second exposure of endotoxin, and TNFα is the most commonly found cytokine indicator of the phenomenon [137]. During the first 24 h in the current experiment, only one 30 h group received endotoxin, but both groups exhibited attenuated inflammatory responses upon bacterial challenge. We know from a previous experiment in a similar model that small changes in tidal volume and PEEP during mechanical ventilation can induce endotoxin tolerance *ex vivo* [173].

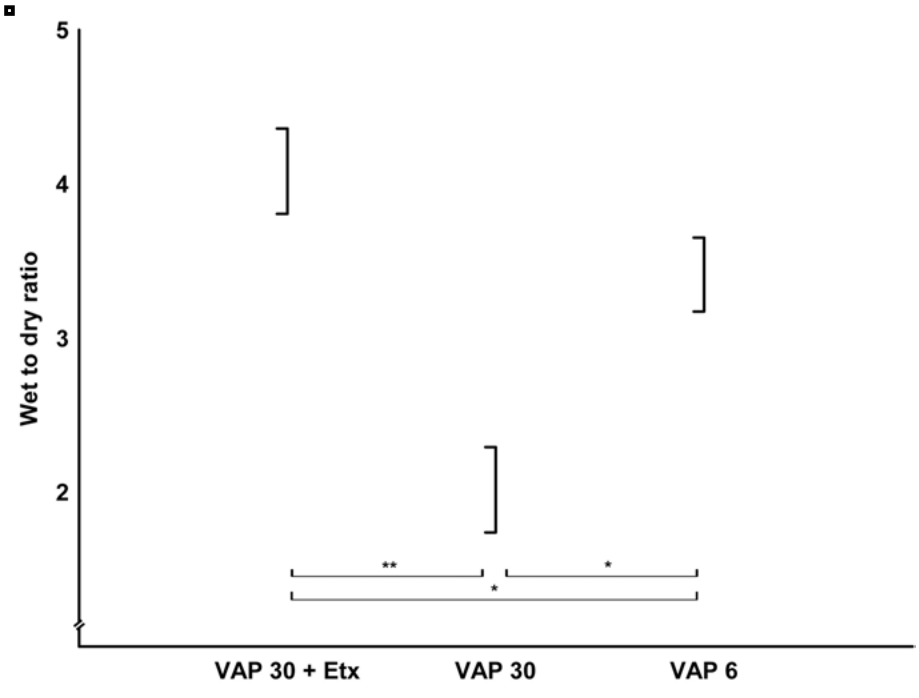

Figure 21. **Wet-to-dry weight ratio of lung tissue**. Mean±SEM, general linear model analysis, * denotes $p<0.05$, ** denotes $p<0.001$.

The clinical relevance of endotoxin tolerance is largely unknown [174]. Seemingly contradictory, mice studies have connected the phenomenon to greater bacterial clearance in the peritoneal cavity [175], while sepsis patients displaying endotoxin tolerance are prone to superinfections [176][4]. Given the bacterial counts in lung tissue in the current model, endotoxin tolerance cannot have been a dominant factor in enhancing clearance from the lungs.

Whether or not influential agents or just correlated, it is notable that nitrite levels in urine and platelets declined in the 30-h groups during the first 24 h and were significantly lower compared to the 6-h group at the time of the bacterial challenge and throughout the experiment. The nitrite levels in urine can be interpreted as indicators of baseline nitric oxide (NO) production and an inducible component from inflammatory mediators [177], and the 30-h groups had lower levels than the 6-h group. NO plays a dual role in *P. aeruginosa* growth where physiologic levels of NO increase growth under anoxic conditions by bacterial denitrification [178], whereas the presence of NO reductase aids intracellular survival by counteracting the host defence systems [179]. The platelet counts were hypothetically lowered by consumption in inflammatory active regions of the lungs in the first 24 h [180].

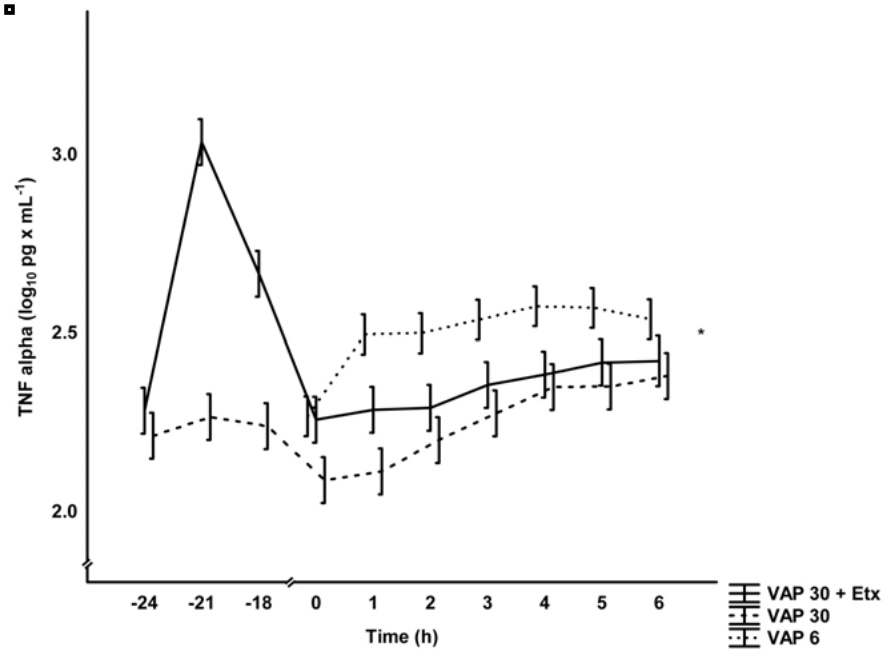

Figure 22. **TNF alpha in plasma**. Mean±SEM, general linear model analysis based on data from 0 to 6 h between all groups, axis scale changes at 0 hours, a) * denotes $p<0.001$, b) p 0.95, c) * denotes $p<0.01$, axis scale changes at 0 hours.

We also present group differences in the physiologic variables. Reasonably, the lungs from the 30-h groups were physiologically different to the unaffected lungs of the 6-h group at the time of the bacterial challenge. The pulmonary airway pressure variables were all highest in the endotoxin treated group. Although not an outcome variable, tidal volume was significantly reduced according to protocol in both 30-hour groups due to lower lung compliance, simultaneously with a decline in PaO_2/FiO_2 ratio during the first 24 hours.

The relatively moderate initial ventilator settings during the first 24 h may well have reduced airway patency, and produced areas of cyclic recruitment and tissue edema [181]. Enhanced clearance of bacteria from the lungs has been associated with the reduction of atelectasis in pigs [162], and the reduction of edema in rats [165]. The wet-to-dry ratio variable, indicating tissue edema likely reflects basic differences in model between the 30-h and 6-h groups. The difference between the 30-h groups is explained by an endotoxin effect on lung tissue edema production in one group [182]. The fact that the 6-h group took an intermediate position indicates that either the edema-producing effect of the bacterial challenge was blunted by 24 h of mechanical ventilation without endotoxin exposure, or that the combined effect of preparatory surgery and a direct bacterial challenge was a relatively more severe inflammatory stimulus in the 6-group than in the 30-h group that was

not exposed to endotoxin. Additionally, it highlights the merely relative impact of tissue edema on bacterial clearance in analogy with endotoxin tolerance. Other factors were of higher importance in bacterial clearance than edema in the 6-h group that displayed the lowest bacterial counts.

The translational relevance of our findings is that they underline the possibility that patients will be variably susceptible to bacterial infection depending on time spent in ventilator and on their inflammatory state. The latter docks to an emerging area of research aiming to subdivide sepsis to find interventions related to inflammatory responsiveness [183].

Conclusions

Mechanical ventilation and systemic inflammation before the onset of pneumonia additively have the potential to enhance *P. aeruginosa* growth in lung tissue. Hypothetically, a reduced bacterial clearance in the lung parenchyma could stem from an acquired attenuation of the host inflammatory response and from induced alterations in lung physiology at the time of the bacterial challenge.

Concluding remarks and future perspectives

The conclusions that were drawn from these experiments are in support of the use of protective ventilation. Protective ventilation is good because it adds less gasoline to the fire. The fact that clinicians did not believe that they could harm patients and continued to use large tidal volumes until the turn of the millennium was indeed an advantage for trialists searching for proof of concept. Today is a different story where the easy marks are gone; clinicians have largely changed their behavior avoiding the large volumes. Scientific experiments like the ones performed for this thesis keep finding support for the main argument, and provide additional incentive to use protective ventilation outside of the ARDS domain. However, the tricky bit will be to move beyond the first step of reducing tidal volume, which admittedly is a macro-level variable. Already, we know that the large problem with an injured lung resides on the micro-level due to heterogeneities within the parenchyma. It is safe to bet that any additional real progress in lung protective ventilation will have to move diagnostics and treatment to the micro-level. This will require bedside monitoring to evaluate ventilation continuously. In this regard, the use of exhaled-breath condensates bears potential promise for early indication of key substances. Sadly, our own efforts in this area expensively crashed and burned. But you can´t make an omelet…

Regarding the kingpin of SIRS 'sepsis', the hot scientific pursuit of one magic bullet to cure them all has lost some momentum. It is repeated sadly over and over. In spite of this mortality seems to have gone down. A highly interesting and necessary future will be to move from the macro-level of 'patient categories' to individual patients. This will require research aiming to define states of inflammation or inflammatory responsiveness, and states of metabolism to tailor treatment to the individual. Additionally, the methods will have to be brought all the way to the twenty-first century bedside. Also in the genetics area efforts will have to be made to assess susceptibility to ventilator and inflammatory injury and to tailor treatment. Slutsky prophesized a future for Ventilogenomics, so why not a suggestion of Septicogenomics. If anything, the clinic will not be less complex.

Swedish summary

Forskning inom det medicinska området är till för att förbättra vården för människor. Ofta behövs experiment för att förklara grundläggande mekanismer vid olika sjukdomstillstånd, eller för att värdera olika behandlingsmetoder. Människor kan inte vara del av experiment som inbegriper farliga moment och därför används i dessa fall **djurmodeller**. Medicinsk forskning med djurmodeller motiveras av nyttan som kunskapen kan ge människor och är reglerad av lagar. Innan ett djurförsök utförs så måste en etiknämnd godkänna försöket.

Respiratorer är datoriserade blåsbälgar som ger andetag till patienter som inte orkar eller kan andas själva. Dessa maskiner förekommer på intensivvårds och operationsavdelningar. Den form av andning som ges är övertrycksandning (som blåser in), vilken skiljer sig från den naturliga undertrycksandningen (som suger in). Det har länge varit känt att man kan skada lungan genom att blåsa upp den hårt, tills den spricker exempelvis. Det är också skadligt för lungan att helt falla ihop, kollapsa, och sedan öppnas igen. Det senare sliter i lungvävnaden och ger inflammation. Den vanligaste formen av lungskada som skapas av en respirator på människor idag uppstår av en blandning av att vissa lungdelar översträcks och andra kollapsar och öppnas med varje andetag. De här negativa effekterna kan motverkas relativt enkelt på patienter med friska lungor genom att man håller lungan helt öppen med ett litet övertryck hela tiden och är varsam med hur stora andetag man ger. Däremot är det mycket svårt att undvika dessa effekter när man behöver använda respirator på patienter som har skadade lungor eller lunginflammation, patientkategorier som är vanliga på intensivvårdsavdelningar. Det beror på att lungvävnaden i dessa fall inte är homogen utan innehåller både luftförande delar och igensatta delar. Respiratorn kan hjälpa patienten att överleva och läka sin sjukdom, men kan också förvärra både lungskadan och ge skador på andra organ än lungorna. Den respiratorbehandling som syftar till att göra så liten lungskada som möjligt brukar kallas för **protektiv ventilation**.

Immunsystemet har till uppgift att försvara kroppen mot angrepp från sjukdomsframkallande organismer som bakterier och virus. Det finns en medfödd del som reagerar snabbt och allmänt mot grundläggande delar av exempelvis bakterier, och en mer riktad del som kan lära sig att känna igen särskilda bakterier eller virus. Immunsystemet agerar till stor del via inflammation som känns igen genom rodnad, svullnad, värme och smärta. Inflammation kan även sättas igång av andra saker än infektion, såsom

kroppsskada från ett fallande piano eller kirurgens kniv. Vid allvarliga tillstånd kan inflammationen, som gör nytta lokalt i infektions eller skadeområdet, sprida sig och skapa ett tillstånd som kallas **systeminflammation**. Systeminflammation är ett tillstånd som ofta leder till organskada och död om det inte kan hävas. Patienter med systeminflammation finns ofta inom intensivvården. Systeminflammation som beror på en infektion kallas för **sepsis** – blodförgiftning. Immunsystemets celler är mycket aktiva under systeminflammation och utsöndrar en mängd ämnen med olika funktioner, exempelvis **cytokiner**. Dessa påverkar inflammationen genom att elda på eller dämpa. Cytokin-nivåer kan användas för att värdera hur stor inflammationen är i olika experiment, och framförallt som mått på att en behandling har effekt på inflammationen.

Det är vanligt att patienter som behandlas i respirator får en ny lunginflammation under sin behandling. Denna kallas för **ventilator-associerad pneumoni** (VAP) och bidrar starkt till att patienter dör. Det är framförallt dåligt att få VAP efter en längre tid i intensivvård, då de bakterier som orsakar infektioner sent i ett intensivvårdsförlopp vanligen är svårbehandlade. ***Pseudomonas aeruginosa*** är exempel på en sådan bakterie.

I de experiment som ingår i avhandlingen så ställer vi frågor om hur protektiv ventilation kan påverka systeminflammation; hur protektiv ventilation påverkar inflammation från enskilda organ; hur protektiv ventilation kan påverka en lunginfektion; och till sist hur tiden med respiratorbehandling och systemisk inflammation innan en lunginfektion påverkar bakterieväxten. Vi har använt oss av en grismodell, med små variationer, till alla experiment. Djuren har varit djupt sövda under hela försöken och avlivats vid försökens slut. Kirurgi utfördes för att lägga in slangar för mätningar och provtagningar. En slang lades i luftstrupen för att kopplas till respiratorn; slangar i blodkärl i halsen lades för att mäta olika hjärtfunktioner samt för provtagning från blod nära levern och hjärnan; en slang lades via buken till portavenen som leder till levern. Systemisk inflammation sattes igång med hjälp av endotoxin, som är en del av vissa bakteriers väggstruktur och mycket effektivt retar immunsystemet. I två experiment användes levande bakterier, *P. aeruginosa*, som gavs direkt in i lungan vid försökens start. Dessa bakterieförsök utvärderades genom bakterieodlingar som gjordes från bitar av lungan. I tre av experimenten jämfördes protektiv ventilation med en kontrollgrupp som hade normala inställningar på respiratorn. Dessa normala inställningar är vanligt förekommande inom sjukvården idag. I ett experiment användes enbart normala respiratorinställningar, och istället var det tiden och inflammationen som skilde grupperna åt.

Resultat. I det första experimentet visade vi att protektiv ventilation dämpar systeminflammationen som vi satt igång med endotoxin. Detta kunde mätas med vissa cytokinnivåer, och vi kunde även se att det fanns mindre skada på hjärtmuskeln. I det andra experimentet visade vi att inflammationen, mätt som cytokinnivåer, ser olika ut i olika organ. Framför

allt såg vi att protektiv ventilation dämpar inflammationen i levern. I det tredje experimentet visade vi att protektiv ventilation minskar antalet bakterier som växer i lungan vid lunginfektion med *P. aeruginosa*. I det fjärde experimentet visade vi att behandling med respirator i 24 timmar minskar lungans förmåga att göra sig av med *P. aeruginosa*. Om vi lade till systemisk inflammation till respiratorbehandlingen försämrades förmågan ytterligare.

Sammanfattningsvis så har vi i experimenten visat att protektiv ventilation kan minska systeminflammation, minska leverns inflammation och minska bakteriebördan vid lunginfektion. Dessutom visade vi att respiratorbehandling i sig, med eller utan systemisk inflammation, påverkar lunginflammation negativt med högre bakterietal. Vår slutsats är att protektiv ventilation har fördelar och ska användas närhelst respiratorbehandling krävs, men att det är bäst att hålla sig borta från respiratorbehandling.

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