Molecular mechanisms of ventilator-induced acute kidney injury: Mechanical ventilation can modulate neutrophil recruitment to the kidney

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1. Introduction

1.1. Acute lung injury/acute respiratory distress sydrome

The first report describing acute respiratory distress syndrome (ARDS) was published in 1967 (Ashbaugh et al. 1967). The American-European Consensus Committee (AECC) recommended new criteria for the definition of ARDS in 1994. According to these new criteria, clinical ARDS is characterised by: (i) acute onset, (ii) bilateral infiltrates on a chest radiograph, (iii) a partial pressure of oxygen in arterial blood (PaO_2):fraction of inspired oxygen (FiO_2) <200 mmHg, and (iv) a pulmonary artery wedge pressure <18 mmHg, or no evidence of left atrial hypertension. Acute lung injury (ALI), a less severe form of respiratory failure, is defined identically except for a higher limiting value of <300 mmHg for the PaO_2:FiO_2 (Bernard et al. 1994, Raghavendran et al. 2008). The pathophysiological manifestations of ALI/ARDS are pulmonary oedema, damage to the cells of the alveolocapillary barrier, infiltration of inflammatory cells, and increased production of inflammatory mediators (Matsuyama et al. 2008). Additional pulmonary manifestations may also be evident, including impaired lung compliance, collapsed alveoli, and ventilation-perfusion mismatch with physiological shunting and increased dead space (Folkesson et al. 1998, Moloney and Griffiths 2004).

Many different factors and diseases can trigger the development of ALI/ARDS. These can be separated into two groups: direct (pulmonary) and indirect (non-pulmonary or systemic) causes. The primary direct insults include pulmonary infection, hyperoxia, gastric aspiration, mechanical ventilation, and pulmonary contusion. Common indirect causes of ALI/ARDS include sepsis, drug overdose, burn injury, trauma, and multiple blood transfusions. Acute lung injury/acute respiratory distress syndrome frequently have systemic components including sepsis and trauma. This is a characteristic of ALI/ARDS that is not present in most other pulmonary diseases. Therefore, the development of new therapeutic agents or strategies which aim to inhibit systemic factors involved in the evolution of ALI/ARDS and multiple organ dysfunction syndrome (MODS) might contribute to the development of a novel therapy (Vincent and Zambon 2006).

The most important risk factors that predict increased mortality in patients with ALI/ARDS are sepsis, the incidence of MODS, the mechanism of lung injury, right ventricular dysfunction, age, organ transplantation, and liver dysfunction; amongst

others (Atabai and Matthay 2002). Additionally, the number of days of mechanical ventilation before manifestation of ARDS is also an independent risk factor for mortality (Monchi et al. 1998).

It was estimated that an annual incidence of ARDS in the United States of America is about 190000 cases, with a mortality of approximately 35% (Rubenfeld et al. 2005). However, reported mortality rates vary from 31-74% which is in accordance with some findings suggesting that the AECC definition underestimates the mortality of ARDS as a result of overestimating the incidence of ARDS. It is currently under discussion whether a new consensus should be developed to better categorise the severity of lung damage in patients with ALI/ARDS (Villar et al. 2007).

The pathophysiological features of ALI/ARDS are presented in Figure 1 and can be portrayed as having three sequential but overlapping phases: an inflammatory phase, a proliferative phase, and a fibrotic phase. The main components of the inflammatory phase involve leukocyte recruitment (neutrophils, alveolar macrophages), oedema formation (accumulation of protein rich fluid with cellular debris in the alveolar airspaces), necrotic or apoptotic alveolar epithelial type I cells, endothelial dysfunction, and surfactant depletion. Additionally, there is increased production of inflammatory mediators, mostly by neutrophils and alveolar macrophages, including interleukins (interleukin (IL)-1, -6, -8, and others), chemokines (chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C-X-C motif) ligand 2 (CXCL2), chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 5 (CCL5)), growth factors (vascular endothelial growth factor (VEGF)), coagulation factors (platelet-activating factor (PAF)) and others. There are also some studies suggesting a role for an anti-inflammatory component that is activated during ALI/ARDS, in particular IL-4 and IL-10.

When the shift from the inflammatory phase to the fibrotic and the proliferative phase occurs, the main pathogenic consequences are the development of fibrotic tissue, alveolar collapse, and matrix reorganisation. The key players of the fibroproliferative phase include transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), IL-4, and IL-13 (Bellingan 2002).

1.2. Mechanical ventilation: definition and classification

Mechanical ventilation was implemented on a large-scale for clinical use in the late 1950's and early 1960's (Oeckler and Hubmayr 2007). Mechanical ventilation is a life-saving intervention required for patients with respiratory failure of various aetiologies including sepsis, ALI/ARDS, and trauma (Dhanireddy et al. 2006).

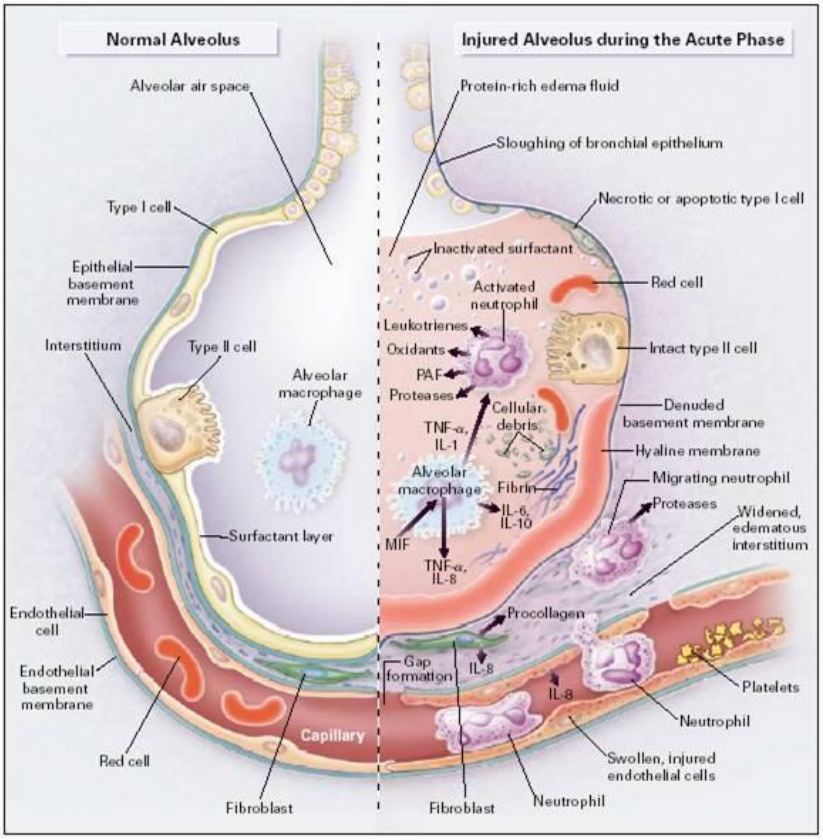

Figure 1. The normal alveolus and the injured alveolus in the acute phase of the acute respiratory distress syndrome. Abbreviations: platelet-activating factor (PAF), macrophage migration inhibitory factor (MIF), tumour necrosis factor (TNF)-α, interleukin (IL) (Ware and Matthay 2000).

The main aims of mechanical ventilation are to maintain adequate gas exchange, facilitate recovery of patients in the intensive care unit (ICU), and to protect the airways following injury, drug overdose, trauma, or major surgery. Inappropriate mechanical ventilation and respiratory support are one of the most critical ICU-related factors leading to poor outcome and increased morbidity and mortality (Agro, Cataldo and Mattei 2009). Mechanical ventilation can contribute to the development of ALI/ARDS, or can worsen pre-existing ALI/ARDS (Jones 2008). Importantly, most ventilated ALI/ARDS patients die **not** from respiratory insufficiency, but from MODS (Del Sorbo and Slutsky 2011).

Mechanical ventilation influences cardiac output, and has many systemic effects on extrapulmonary organs. To avoid the development of ventilator-induced lung injury (VILI) and resultant MODS, appropriate ventilator settings - which are currently a topic of heated debate - must be employed.

Maintenance of appropriate gas exchange is the key objective of mechanical ventilation. However, to reduce the effect of oxygen toxicity (when too high concentrations of oxygen are applied), ventilation strategies must be carefully

optimised. In clinical management it is standard practice to fix the FiO_2 below 0.6 to limit the impact of oxygen toxicity on the tissues. Increasing the inspiratory/expiratory ratio and higher airway pressures, and the use of recruitment manoeuvres, can further promote adequate oxygenation.

Another critical concern in mechanical ventilation of patients is hypercapnia. The reduction of airway pressures and tidal volumes used to avoid VILI can lead to increased levels of carbon dioxide (CO_2) in the lung, due to inappropriate gas exchange. The consequences of hypercapnia include respiratory acidosis, increased cardiac output and neurological changes that have to be managed in clinical practice (Cordingley and Keogh 2002). In 1990 it was suggested that prolonged hypercapnia (permissive hypercapnia) and a reduction in airway pressure was correlated with decreased mortality of ALI/ARDS patients, suggesting the beneficial use of permissive hypercapnia together with a protective mechanical ventilation strategy in critical care practice (Hickling, Henderson and Jackson 1990).

The “open lung” concept was proposed to describe the behavior of the lung during lung injury in order to potentially treat ALI/ARDS. The “open lung” was defined by optimal oxygenation and recruitment of all alveoli and a reduction of shear forces. That is why in ALI/ARDS patients one of the strategies proposed to open atelactatic areas of the lung is the use of sufficient positive end-expiratory pressure (PEEP) (Haitsma and Lachmann 2006).

The application of PEEP prevents alveoli from collapse, contributes to better oxygenation by increasing the surface area for gas exchange, and preserves surfactant function. One of the principle challenges during mechanical ventilation of ALI/ARDS patients, is to fully recruit the ventilated lung. The pressure applied should not provoke cell stress or overdistension of alveoli. One of the techniques employed to improve lung recruitment is to apply sufficient PEEP. It remains under debate which level of PEEP can be considered to be protective in ALI/ARDS patients. In general, it was proposed that “high” PEEP levels, ranging from 10-15 cmH_2O improve oxygenation, although no improvement in mortality, ventilator-free days and the development of MODS were observed (Prabhakaran 2010).

Alternative means of lung recruitment during mechanical ventilation include prolonging the inspiratory time, and the use of recruitment manoeuvres. Increases in inspiratory time serve as an alternative to PEEP, when the plateau pressure reaches 35 cmH_2O. Air trapping in the lung can also occur when the expiratory time is too short (Marik 2010).

Recruitment manoeuvres also constitute part of the therapy for ALI/ARDS patients. Although there is no clear evidence that recruitment manoeuvres have

beneficial effects on outcome and the duration of a hospital stay, it was observed that these manoeuvres can improve oxygenation (Hodgson et al. 2009).

There are two principal modes used to control mechanical ventilation: volume and pressure. It has been debated over the past years which of these two mechanical ventilation control mechanisms are of more benefit to patients with respiratory insufficiency: the pressure-controlled or volume-controlled mode. In the pressure-controlled mode, a predetermined pressure is delivered to the airways until this set pressure is reached in the lungs. For the volume-controlled mode, a predetermined tidal volume regulates the volume of gas inspired. Recent consensus considers the pressure-controlled mode as a protective strategy over the volume-controlled mode. Comparing these two types of ventilation strategies, the pressure-controlled mode reduces the risk of lung injury and the work of breathing, and allows for homogenous gas distribution and tidal volume across the entire lung. There is also a decrease in peak airway pressure in the pressure-controlled mode which limits overdistension of the lung, and thus, barotrauma. In present study the pressure-controlled mode of mechanical ventilation was selected over the volume-controlled mode (Prella, Feihl and Domenighetti 2002).

The development of VILI is one of the main consequences of inappropriate ventilator settings. In the past, “high” tidal volumes ranging from 10 ml/kg to 15 ml/kg were applied. This provoked or worsened ALI, rather than facilitated adequate gas exchange. In 2000, the ARDSNet proposed to reduce tidal volumes to 6 ml/kg in accordance with plateau pressure, which should be less than 30 cmH_2O. It was observed in a large clinical trial that this protective ventilation strategy reduced mortality and number of ventilator days of ARDS patients (ARDSNet 2000).

It has been demonstrated that mechanical ventilation of healthy rat lungs can lead to the development of VILI, when high peak inspiratory pressure (PIP), high tidal volumes, or low PEEP are applied (Dreyfuss et al. 1988). Interestingly, in an experimental model of mechanical ventilation of healthy mice, even clinically-relevant ventilator settings for tidal volume (7.5 ml/kg) compared with a more injurious ventilation with 15 ml/kg, at constant PEEP (2 cmH_2O), were able to induce VILI, suggesting a strong impact of mechanical ventilation on lung pathophysiology (Wolthuis et al. 2009). The mechanisms of injurious mechanical ventilation which cause lung injury remain under consideration, however, inflammation and coagulation have been demonstrated to play a role in rodent models, including the production of cytokines and other mediators such as IL-1β, IL-6, Ccl2, prostaglandin-endoperoxide synthase 2, plasminogen activator inhibitor (PAI)-1 and tissue factor (TF) (Ma et al. 2005).

Mechanical ventilation is one of the first techniques used in critically ill patients when breathing support is required. As illustrated in Figure 2, inappropriate ventilator settings can lead to development of ALI/ARDS or aggravate pre-existing ALI/ARDS. The injured lungs can also affect the extrapulmonary organs, leading to the development of MODS (Pinhu et al. 2003).

1.3. Multiple organ dysfunction syndrome

Pulmonary dysfunction in the onset of ALI precedes damage to other distant organs such as the kidney, liver and heart in trauma patients (Ciesla et al. 2005). Injurious mechanical ventilation can augment ALI/ARDS and is associated with an increased inflammatory response including elevation of immune cell number, reactive oxygen species (ROS) production, cell apoptosis, and changes in the levels of mediators of inflammation (Manicone 2009). In the present study, the kidney was considered as an organ affected early in MODS which developed in response to injurious mechanical ventilation. There is a harmful interaction between mechanical ventilation and ALI, and the presence of acute kidney injury (AKI). Several recent studies have explored more basic, molecular mechanisms of the complex cross-talk between injured organs in patients admitted to the ICU (Koyner and Murray 2010). Three mechanisms have been proposed to describe lung-kidney interactions during the pathogenesis of ALI/ARDS and the development of MODS. The impairment in gas supply and blood flow in distant organs together with a pulmonary inflammatory reaction that causes spill-over of mediators into the systemic circulation are generally regarded as the main factors triggering MODS.

There is ongoing debate concerning how blood oxygenation can affect distant organs, particularly the kidney. It is widely believed that hypoxaemia, a decreased blood PaO_2, can trigger renal insufficiency, probably by activation of ROS production (Kim et al. 2009) together with an imbalance in nitric oxide and the vasodilator/vasoconstrictor machinery. As a result of poor oxygenation, disturbances to the microcirculation and microvascular dysfunction in the kidney were considered to be major factors leading to renal insufficiency. Therefore, the imbalance in oxygen supply and oxygen utilisation by cells is thought to be a crucial mechanism that should be targeted in the management of AKI (Legrand et al. 2008).

Changes in carbon dioxide distribution also play a central role in pathogenesis of MODS, where hypercapnia can lead to a reduction in renal blood flow and increased renal vascular resistance (Hemlin et al. 2007).

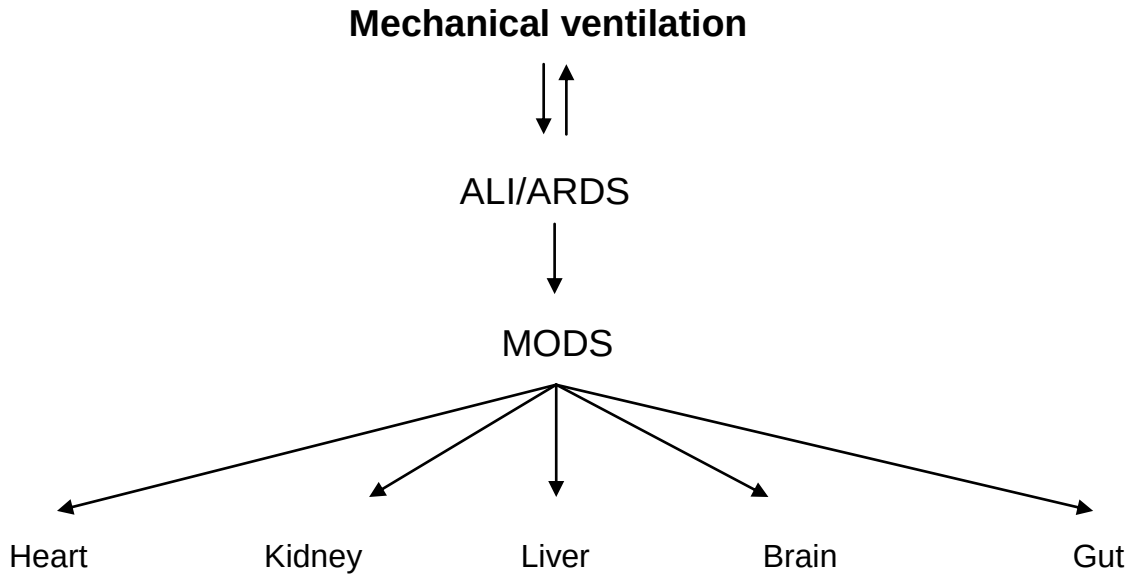

Figure 2. Mechanical ventilation can contribute to the development of multiple organ dysfunction syndrome. Mechanical ventilation can provoke the development of acute lung injury/acute respiratory distress syndrome (ALI/ARDS). Consequently, the injured lung may communicate with distant organs causing them to fail with multiple organ dysfunction syndrome (MODS).

The mechanisms underlying these effects are not clear, but the stimulatory effect of carbon dioxide on noradrenaline release, with subsequent action on the sympathetic nervous system and the renin-angiotensin-aldosterone system are regarded as important factors in the pathogenesis of renal damage. Importantly, the decrease in renal blood flow also occurs in normoxic hypercapnia, suggesting a central role for $PaCO_2$ levels in the determination of the renal blood flow, rather than PaO_2 (Kuiper et al. 2005).

Mechanical ventilation tends to depress cardiac output by increasing pulmonary vascular resistance due to the compression of pulmonary vessels, and by affecting blood volume in the lung by decreasing preload and increasing right ventricular afterload (Bercker et al. 2009, Vieillard-Baron et al. 1999). It is known that decreased cardiac output during mechanical ventilation by positive pressure provokes changes in kidney function, and leads to a decrease in renal perfusion, glomerular filtration rate, and urinary output. Therefore, monitoring the haemodynamics of patients, the mean arterial pressure (MAP) in particular, is important to prevent the development of

ischaemia in distant organs. Another study demonstrated that mechanical ventilation of healthy rats with a high tidal volume of 27 ml/kg reduced glomerular filtration rat compared to a low tidal volume of 8 ml/kg, however, no information regarding mechanism was provided (Luque et al. 2009).

Kidney function can be also compromised by biotrauma. In this case, mechanical ventilation damages the lungs which then release mediators into the systemic circuit. Afterwards, these mediators can influence distant organs causing damage to cells and impairing organ function (Koyner and Murray 2008).

As demonstrated in Figure 3, mechanical ventilation can trigger biophysical (such as cyclic stretch and ovedistention) and biochemical (such as release of mediators of inflammation) injury to the lungs. This injury can lead to a systemic and distal organ response, which ultimately can develop into MODS. Many studies have demonstrated the relationship between ALI/ARDS and the development of MODS. Some of these studies have investigated the molecular mechanisms that link ALI/ARDS with systemic responses, while other studies have concentrated rather on physiological aspects of the progression from ALI/ARDS to MODS. The mechanisms of MODS at play in ALI/ARDS patients may include the induction of apoptosis and necrosis in distant organs, activation of systemic inflammation, which encompasses modulation and proliferation of immune cells, the release of cytokines or other mediators, and changes in the endothelium (Luh and Chiang 2007).

Biotrauma and lung-kidney interactions have been addressed in several studies performed in experimental animal models, and in ventilator-supported patients (Jaecklin, Otulakowski and Kavanagh 2010). It is known that the levels of many acute-phase proteins, including cytokines, are elevated in the lungs and in broncho-alveolar lavage fluid (BALF) during ALI/ARDS. These cytokines can then be released into the systemic circulation, and are thus implicated in the development of MODS, and serve as predictors of outcome. A study on 27 patients was performed to assess the relationship between pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1, IL-6 and IL-8 and the course of ALI/ARDS and MODS. These findings revealed that patients with increased levels of IL-1 and IL-6 which did not decline over time exhibited poor prognosis and poor outcome (Meduri et al. 1995). It was also demonstrated in other studies that the mechanical stress caused by mechanical ventilation leads to the induction of cytokine release. Importantly, some stress can also be induced by low tidal volume ventilation (Meier et al. 2008).

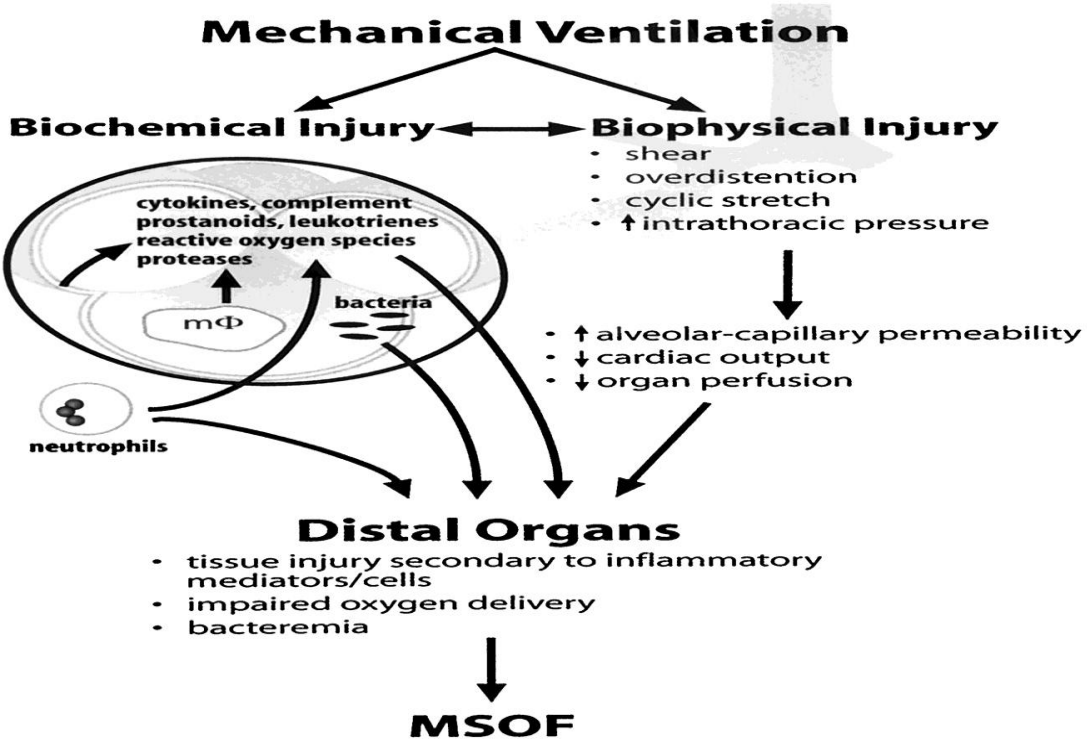

Figure 3. Mechanisms by which mechanical ventilation contributes to the development of multiple organ dysfunction syndrome. Mechanical ventilation can provoke biochemical and biophysical injury to the lungs and in consequence may spread the action to distal organs, causing the development of multi organ dysfunction syndrome (MODS). Abbreviations: multi-system organ failure (MSOF), alveolar macrophage (mΦ) (Slutsky 1999).

Some studies have demonstrated that 12 h of injurious mechanical ventilation, compared with non-injurious mechanical ventilation, led to increased release of MODS markers, notably creatinine and aspartate aminotransferase, and the occurence of apoptotic events (determined by increased production of soluble Fas ligand). This suggested that mediators contributing to distant organ apoptosis may be potential targets for the treatment of mechanically ventilated ARDS patients (Imai et al. 2003).

Mechanical ventilation leads to activation of the endothelium and to inflammatory responses in both the lungs as well as extrapulmonary organs. Several studies have documented the systemic effects of mechanical ventilation of healthy animals without pre-existing lung injury. Recently, it was demonstrated that ventilation of mice without pre-existing lung injury, with PIP of 20 cmH_2O and a PEEP of 0 cmH_2O leads to a time-dependent increase in the expression of chemokines and cytokines, and increased recruitment of granulocytes to the lungs and distant organs (Hegeman et al. 2009).

Multiple organ dysfunction syndrome can also be induced by a combination of mechanical ventilation and lipopolysaccharide (LPS) administration. In one study, increased levels of markers of renal and hepatic damage and increased release of

plasma cytokines were noted, while no apoptotic events were observed. It is worth mentioning that in that study, mechanical ventilation alone did not cause organ damage, but was able to induce the release of cytokines and chemokines into the plasma (O'Mahony et al. 2006). Another study demonstrated that low tidal volume (6 ml/kg) mechanical ventilation did not attenuate the development of acute kidney injury (AKI) compared to high tidal volume (10 ml/kg) mechanical ventilation in ICU patients without ALI at the onset of mechanical ventilation as assessed by biomarkers of AKI (Cortjens et al. 2011).

Additionally, 4 h of mechanical ventilation of healthy mice with two different ventilation strategies such as protective, low tidal volume (6 ml/kg), or injurious, high tidal volume (17 ml/kg) did not cause inflammatory responses in distal organs. Neither ventilation strategy influenced IL-6 or TNF-α levels in systemic organs, except for VEGF concentration which was increased in the liver and kidneys. This was not the case for animals pre-injured by acid aspiration, followed by mechanical ventilation to provoke ALI/ARDS. Ventilation of lungs pre-injured by acid aspiration with injurious ventilation strategies caused remote organ inflammation, while the non-injurious ventilation strategy was able to attenuate renal and hepatic inflammation as assessed by IL-6 and VEGF receptor-2 (VEGFR2) measurements. It was then suggested that mechanical ventilation strategies may influence the degree of distal organ injury and inflammation. Therefore, it was proposed that extrapulmonary organs are susceptible to a harmful influence of mechanical ventilation (Gurkan et al. 2003).

Taken together, these observations all indicate that MODS can be induced by mechanical ventilation. Injurious mechanical ventilation strategies lead to the development of MODS, while protective ventilatory support has less impact on remote organ. Nevertheless, additional studies have to be performed to understand the mechanisms of lung-kidney crosstalk and the influence of mechanical ventilation on the manifestation and progression of MODS. Work presented in this thesis aimed to begin addressing this question.

1.4. **Acute kidney injury**

The main function of the kidney is to excrete waste and toxins, although the role of the kidney in maintaining electrolyte balance, pH, and fluid balance should not be forgotten. The kidneys receive approximately 25% of the cardiac output, and any disturbances to the systemic circulation affect renal blood flow. Acute kidney injury is

associated with limitation of kidney function, including a decreased glomerular filtration rate, and a reduced capacity to regulate the volume of body fluids, and acid-base balance. The classification of AKI describes prerenal (such as low cardiac output, hypovolaemia), intrarenal (such as toxic agents and nephrotoxic mediators, acute tubular necrosis) and postrenal (such as a tumour of the ureter) causes of disease (Needham 2005).

One of the first descriptions of the pathology of acute renal failure was published in 1941 (Bywaters and Beall 1998). Insults such as sepsis, trauma, respiratory failure, as well as some medications can trigger AKI (Paladino, Hotchkiss and Rabb 2009). Acute kidney injury is classified according to the RIFLE (Risk, Injury, Failure, Loss, End-stage kidney disease) criteria and is associated with the development of MODS. It was estimated that 30% of patients admitted to the ICU suffer from AKI, and kidney dysfunction has a negative impact on patient morbidity and mortality (Faubel 2009).

The overall mortality of critically ill patients with AKI is 45.8% *versus* 25.7% for patients without AKI (Ostermann, Chang and Group 2008). Recent studies have demonstrated that renal therapy in patients with AKI admitted to the ICU did not improve outcome or recovery of renal function, or reduce the dysregulation of other distant organ function (Palevsky et al. 2008).

Inflammation and vascular processes are the main factors contributing to the onset of AKI. Recruitment and migration of immune cells, release and activity of cytokines and growth factors, and cell death, are the main pathological manifestations of AKI.

In animal models of AKI, the primary insult results in endothelial activation and necrosis or apoptosis of epithelial cells. The release of inflammatory mediators such a chemokines (such as CXCL1, CCL2, CCL5) by activated cells recruits immune cells including neutrophils, macrophages and T-cells to the site of inflammation. The inflammatory cells release cytokines such as IL-6, TNF-α, interferon (IFN)-γ, which serve as intercellular comunicators. The genes and proteins involved in adhesion (p-selectin, inter-cellular adhesion molecule (ICAM)-1), the complement system, and recognition (toll-like receptor (TLR)-4) are also upregulated in acute AKI. The activation of proinflammatory pathways results in the migration of leukocytes into the kidney (Akcay, Nguyen and Edelstein 2009).

The activation of the immune system occurs very early in inflammatory and infectious diseases, and the involvement of neutrophils, T-cells and macrophages is well documented in the pathogenesis of AKI. Neutrophils which respond rapidly to injury contribute to tissue damage, releasing proteases and other enzymes stored in

neutrophil granules, thereby generating ROS, and leading to cell death and structural damage.

Macrophages function as the cells which limit infection and tissue injury, and promote wound healing. It was demonstrated in a renal ischaemia-reperfusion rat model that macrophages exhibit anti-inflammatory effects, reducing tissue damage *via* haeme oxygenase 1 (Hmox1) action (Gueler et al. 2007). However, through hyper-activation, macrophages can have a detrimental effect on tissue, leading to tissue damage (Laskin et al. 2011). Macrophages, when hyper-activated, release pro-inflammatory cytokines and other mediators, which then exacerbate the inflammatory processes. The T-cells are the initial components engaged by the adaptive immune system. Studies performed on nude mice (which lack T-lymphocytes) demonstrated reduced AKI compared to wild-type control mice (Kinsey, Li and Okusa 2008).

Although the pathophysiology of AKI has been extensively studied, the clinical management of AKI is severely hindered since this acute syndrome cannot be detected early enough, largely because early biomarkers have not been identified. Existing markers, such as serum creatinine and blood urea nitrogen (BUN) emerge very late in response to injury and lead to delayed diagnosis of AKI (Waikar and Bonventre 2007). The absence of sensitive and specific biomarkers for the early detection of AKI has impaired progress in the diagnosis and treatment of patients with AKI. Recently, some very early markers of AKI have been proposed and are currently under validation. These new candidate include lipocalin-2 and kidney injury molecule (Kim) 1.

1.5. Biomarkers of acute kidney injury

Acute kidney injury remains a disease with poor outcome. The lack of early biomarkers and early diagnostic criteria are one of the key clinical barriers to the development of potential therapeutic strategies (Melnikov and Molitoris 2008). Several studies have attempted to identify and then evaluate specific and sensitive markers of AKI. Microarrays followed by proteomic analysis of human and eperimental animal material has facilitated the identification of novel pathways, candidate biomarkers and drug targets in AKI (Devarajan 2008). The most promising biomarkers of AKI according to some studies are lipocalin-2, Kim1, IL-18, cystatin C, clusterin, fatty acid–binding protein (FABP), and osteopontin (Vaidya, Ferguson and Bonventre 2008a). In this study, an evaluation of lipocalin-2 and Kim1 as potential biomarkers was attempted. Both lipocalin-2 and Kim1 represent sequentially expressed biomarkers, where lipocalin-2 appears at the onset of AKI, and Kim1 at later time-points (Nguyen and

Devarajan 2008). Additionally, it was demonstrated that lipocalin-2 and Kim1 were also among the genes to be the most up-regulated in at least three separate microarray studies on AKI (Devarajan 2007, Yuen et al. 2006).

Lipocalin-2, which is also called neutrophil gelatinase-associated lipocalin (NGAL), is a glycoprotein that belongs to the lipocalin protein family, a group of extracellular proteins (Flower 1996). Lipocalin-2 is a 25 kDa monomer, which can also exist as a homodimer (appearing at a molecular mass of about 46 kDa) or a 135 kDa heterodimer associated with matrix metallopeptidase (MMP)-9 (Tong et al. 2008). Lipocalin-2 is expressed in neutrophils, but additionally can be found in several other tissues, including the lung, kidney, and macrophages (Wang et al. 2007). In general, lipocalin-2 can act as an iron-carrier protein during bacterial infections, is important for innate immunity, contributes to regulation of inflammation as an acute phase protein, has some apoptotic properties, and is thought to be an AKI biomarker (Tong et al. 2005).

The glycoprotein Kim1 is also regarded as a novel AKI marker (Prozialeck et al. 2007), and is a member of the superfamily of immune regulating adhesion proteins. Structurally, Kim1 possesses a six-cysteine immunoglobulin-like domain and a mucin domain in the extracellular region. Kim1 is also known as hepatitis A virus cell receptor (Havcr)-1 due to its homology to a monkey gene which encodes a protein that was described to be a receptor for hepatitis A virus. Kim1 is believed to contribute to renewal processes and regeneration of kidney function after ischaemia-reperfusion injury (Ichimura et al. 1998).

Recently, several studies were undertaken to evaluate the sensitivity, specificity, and prognostic capacity of novel biomarkers of AKI. In one of these studies, nine urinary biomarkers were tested. Patients with AKI were selected according to RIFLE criteria. The additional non-AKI groups included healthy volunteers, patients admitted to the ICU, and patients undergoing cardiac catheterisation. The main finding of these studies was that among the nine biomarkers evaluated, lipocalin-2, Kim1, hepatocyte growth factor (HGF) and total protein - individually and in combination - are the best indicators of AKI (Vaidya et al. 2008b).

Additional studies were performed on patients who underwent cardiopulmonary surgery. Urine samples were collected 2 h, 24 h and 48 h post-cardiopulmonary bypass (CPB). The 2-h time-point was the most meaningful for the diagnosis of AKI. Among six biomarkers tested, Kim1 displayed the best performance as a biomarker of AKI, 2 h post-CBP (Liangos et al. 2009).

Studies carried out on kidney allograft recipients, however, have demonstrated that lipocalin-2 levels in serum are elevated in these patients compared to the control

group. The authors concluded that lipocalin-2 can also be regarded as a potential early biomarker of damaged kidneys (Malyszko, Malyszko and Mysliwiec 2009).

In animal models, Kim1 was found to be a potential AKI biomarker in models of nephrotoxicity (Ichimura et al. 2004), ischaemia-reperfusion (Vaidya et al. 2006), and proteinuric renal disease (van Timmeren et al. 2006).

In conclusion, there is an urgent need to establish novel markers for AKI to facilitate prompt clinical management of this syndrome. Lipocalin-2 and Kim1 are currently under validation and seem to be a potentially helpful in the diagnosis of AKI.

1.6. Hypothesis and aims of this study

The hypothesis of this study was that inappropriate mechanical ventilation can lead to the development of AKI in a rat model, and that there is communication between mechanically ventilated lungs and the kidneys of ventilated rats.

Multiple organ dysfunction syndrome is a leading cause of mortality in ventilated patients with ALI and ARDS. The kidney is amongst the first of the extrapulmonary organs to be affected by organ failure in mechanically ventilated ALI/ARDS patients. Therefore, this study was performed to explore the communication between the lungs and kidneys during mechanical ventilation of healthy rats. Particular attention was paid to the impact of mechanical ventilation on new, emerging biomarkers of AKI: lipocalin-2 and Kim1. Specifically:

1) Healthy rats were mechanically ventilated with three different ventilation strategies with a pressure-controlled mode (PIP/PEEP = 10/2 cmH_2O, 15/2 cmH_2O or 20/2 cmH_2O) for four or eight hours. Lung injury was assessed by protein content of BALF.
2) The levels of potential biomarkers of AKI were assessed in the kidneys and urine of ventilated animals.
3) The degree of inflammation, as a one of the first manifestations of AKI, was examined in renal tissue at a molecular and histopathological level.

2. Materials and Methods

2.1. Materials

2.1.1. Equipment

ABI PRISM 7500 Sequence Detection System	Applied Biosystems, USA
Block heater	Peqlab, Germany
Catheter 22G	BD, The Netherlands
Developing machine; X Omat 2000	Kodak, USA
Electrophoresis chambers	Bio-Rad, USA
Film cassette	Sigma-Aldrich, Germany
Filter Tip FT: 10, 20, 100, 200, 1000	Greiner Bio-One, Germany
Filter units 0.22 µm syringe-driven	Millipore, USA
Freezer -20 °C	Bosch, Germany
Freezer -40 °C	Kryotec, Germany
Freezer -80 °C	Heraeus, Germany
Fridge +4 °C	Bosch, Germany
Fusion A153601 Reader	Packard Bioscience, Germany
Gel blotting paper 70 × 100 mm	Bioscience, Germany
Glass bottles: 250, 500, 1000 ml	Fischer, Germany
GS-800TM Calibrated Densitometer	Bio-Rad, USA
Light microscope Olympus BX51	Olympus, Germany
Mini spin centrifuge	Eppendorf, Germany
Microtom Micron cool-cut HM355S	Micron, Germany
Multifuge centrifuge, 3 s-R	Heraeus, Germany
Multipette® plus	Eppendorf, Germany
Nanodrop®	Peqlab, Germany
Nebuliser	Penn-Century, USA
PCR-thermocycler	MJ Research, USA
pH/blood-gas analyser	Radiometer, Denmark
Pipetboy	Eppendorf, Germany
Pipetman: P10, P20, P100, P200, P1000	Gilson, France
Power Supply; Power PAC 300	Bio-Rad, USA
Pipette tip: 200 µl, 1000 µl	Sarstedt, Germany
Pipette tip: 10 µl, 20 µl, 100 µl	Gilson, USA

Radiographic film X-Omat LS	Sigma-Aldrich, Germany
Serological pipette: 5 ml, 10 ml, 25 ml, 50 ml	Falcon, USA
Test tubes: 15 ml, 50 ml	Greiner Bio-One, Germany
Thermo-Fast® 96 PCR Plate	Thermo Scientific, USA
Western Blot Chambers: Mini Trans-Blot	Bio-Rad, USA
Ventilator Servo 300	Siemens, Sweden
Vortex machine	Eppendorf, Germany

2.1.2. Reagents

3,3´-Diaminobenzidine tetrahydrochloride	Sigma-Aldrich, Germany
Acetic acid (glacial)	Merck, Germany
Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
Agarose	Invitrogen, UK
Albumine, bovine serum	Sigma-Aldrich, Germany
Ammonium persulfate	Promega, Germany
β-mercaptoethanol	Sigma-Aldrich, Germany
BCA Protein Assay Kit	Thermo Scientific, USA
Bromophenol blue	Sigma-Aldrich, Germany
Buprenorphine	Merck, The Netherlands
Complete protease inhibitor	Roche, Germany
Diaminobenzidine	Sigma-Aldrich, Germany
DEPC water	Roth, Germany
DNA Ladder (100 bp, 1 kb)	Promega, USA
Dithiothreitol	Promega, USA
Dulbecco's 10× PBS without Ca & Mg	PAA Laboratories, Austria
Ethylendinitrilo-*N, N, N', N',* -tetra-acetic-acid	Promega, USA
Ethylene glycol-bis (2-amino-ethylether)-*N,N, N', N'*-tetraacetic-acid	Sigma-Aldrich, Germany
Dulbecco's phosphate buffered saline 10×	PAA Laboratories, Austria
Dulbecco's phosphate buffered saline 1×	PAA Laboratories, Austria
NGAL ELISA kit	Bioporto, Denmark
Eosin	Merck, Germany
Ethanol absolute	Riedel-de Haën, Germany
ECL Plus Western Blotting Detection System	Amersham Biosciences, UK
Ethidium bromide	Roth, Germany
Glycine	Roth, Germany

Glycerol	Merck, Germany
Haematoxylin	Merck, Germany
Heparin	Leo Pharma, The Netherlands
Hydrochloric acid	Sigma-Aldrich, Germany
Hydrogen peroxide 30%	Merck, Germany
Isoflurane	Pharmachemie, The Netherlands
Isopropanol	Sigma-Aldrich, Germany
Methanol	Fluka, Germany
Non-fat dry milk powder	Roth, Germany
Normal rat serum	Harlan, Germany
N,N,N',N'-tetramethyl-ethane-1,2-diamine	Bio-Rad, USA
Pancuronium bromide	NV Organon, The Netherlands
Paraformaldehyde	Sigma-Aldrich, Germany
PCR Nucleotide Mix	Promega, USA
Pertex (Mounting Medium)	Medite, Germany
Precision Plus ProteinTM Standards	Bio-Rad, USA
Quick StartTM Bradford Dye Reagent	Bio-Rad, USA
Pentobarbital	Ceva Sante Animale, The Netherlands
Random hexamers (50 µM)	Applied Biosystems, USA
RNase inhibitor	Applied Biosystems, USA
RNaseZAP®	Sigma-Aldrich, Germany
Rotiphorese Gel 30	Roth, Germany
Roti®-Quick-Kit	Roth, Germany
Sodium azide	Merck, Germany
Sodium chloride	Merck, Germany
Sodium dodecyl sulfate	Promega, USA
Sodium hypochlorite solution 12%	Roth, Germany
Sodium *ortho* vanadate	Sigma-Aldrich, Germany
Sodium phosphate	Sigma-Aldrich, Germany
SuperSignal® West Pico substrate	Thermo Scientific, USA
SYBR® Green PCR kit	Invitrogen, UK
Tween 20	Sigma-Aldrich, Germany
Tris	Roth, Germany
Triton X-100	Promega, USA

2.2. Experimental protocol

Rats were divided into three groups, which were then subjected to three mechanical ventilation strategies. Two time-points were selected: 4 h and 8 h as outlined in Figure 4. Furthermore, one group of non-ventilated, non-instrumented animals were employed to assess a baseline level.

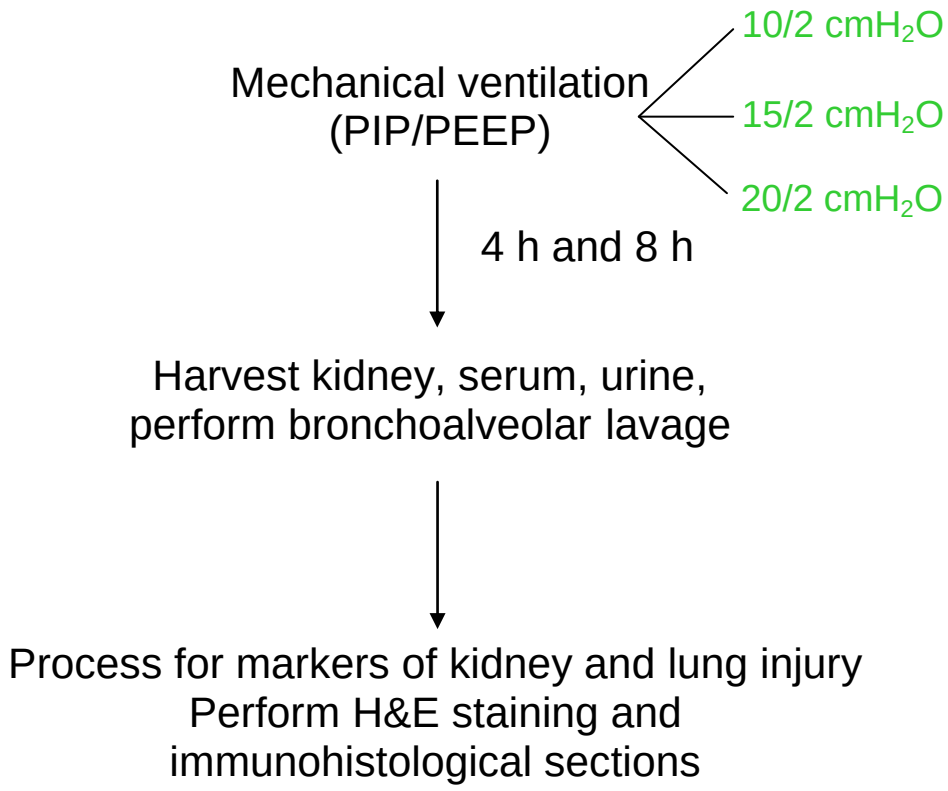

Figure 4. Experimental protocol. Healthy rats were ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). Assessment of candidate biomarkers of AKI, haematoxylin and eosin (H&E) staining, and immunohistological visualisation of immune cells were performed on material from two experimental time-points – 4 h and 8 h. Abbrevations: peak inspiratory pressure (PIP), positive end-expiratory pressure (PEEP).

2.3. Methods

2.3.1. Animals

All procedures were approved by the experimental animal committee of the Erasmus Medical Center (Rotterdam, the Netherlands). All animals were handled in strict accordance with European Community Guidelines. Tissue samples were obtained from specific pathogen-free male rats weighing 350-400 g Sprague Dawley (Harlan, Horst, NL). From rats under anaesthesia, BALF, plasma and urine were collected and all rat

tissues were surgically excised and frozen in liquid nitrogen for further analyses. The animals were housed in individually ventilated cages and were maintained in day/night 7 am-7 pm. The animals received water and food *ad libitum*.

2.3.2. Mechanical ventilation and tissue processing

Rats were anaesthetised after orotracheal intubation under gaseous anaesthesia (60% oxygen: 3% isoflurane), using a miniature nebuliser. Anaesthesia and muscle relaxation were maintained with hourly intraperitoneal (*i.p.*) injections of pentobarbital sodium (stock 60 mg/kg, 17 mg/kg/h), and intramuscular (*i.m.*) injections of pancuronium bromide (stock 2 mg/kg, 0.3 mg/kg/h), respectively. All animals were ventilated in a pressure-controlled mode with PIP of 12 cmH_2O, PEEP of 2 cmH_2O, FiO_2 of 0.3, frequency of 30 breaths/min, and inspiratory/expiratory time (I:E) ratio of 1:2. In order to stabilise the rats haemodynamically, rats received an infusion of 1.5-2 ml/kg/h of 0.9% NaCl into the tail vein. Body temperature was maintained at 37 °C with heating pad. After a 15-min stabilisation, arterial blood gases were taken using a carotid artery catheter and PIP was adjusted according to the ventilation group to which the animals had been allocated. The rats were randomised to one of three ventilator strategies and ventilated for 4 h or 8 h: 1) high PIP/PEEP group, where PIP=20 cmH_2O and PEEP=2 cmH_2O; 2) moderate PIP/PEEP, where PIP=15 cmH_2O and PEEP=2 cmH_2O; and 3) low PIP/PEEP group, where PIP=10 cmH_2O and PEEP= 2 cmH_2O. Non-ventilated animals served as baseline level group. Arterial blood-gas determinations were performed every hour (every 2 h for the 8-h group) using a pH/blood-gas analyser. Mean arterial blood pressure (MAP) was monitored using an intra-arterial carotid artery catheter, every 1 h. After the experiment was completed, all animals were sacrificed by exsanguination. All experiment were carried out to maintain typical reference O_2, CO_2 (35-45 mmHg), pH (7.35-7.45), and MAP (>80 mmHg) values. In order to do that, handling with ventilator (such as frequency of breathing, I:E ratio) and equipment (such as length of intubation tube) settings were managed.

2.3.3. Bronchoalveolar lavage fluid collection

After the rats were sacrificed, the lungs and kidney were removed. Immediately, BALF was obtained by rinsing the airways 3× with normal saline (30 ml/kg, heated to 37 °C). Bronchoalveolar lavage fluid was centrifuged (300 × *g* for 10 min at 4°C) and the supernatant was stored at -80 °C until further processing.

2.3.4. RNA isolation

Isolation of RNA from kidney tissue was performed according to the manufacturer's instructions provided with Roti®-Quick-Kit with some user's modifications.

2.3.5. Determination of RNA concentration

The concentration of isolated RNA was determined according to a protocol from Peqlab by applying 1.5 µl of the sample to a Nanodrop® spectrophotometer.

2.3.6. RNA agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate and analyse the quality of RNA isolated from rat kidneys and to estimate the size of amplicons generated during qPCR reaction. Agarose was mixed with 1× Tris-acetate-EDTA (TAE) buffer and 0.5 µg/ml ethidium bromide. The samples were mixed with 6× DNA loading buffer and loaded onto 2% agarose gel. Electrophoresis was performed at 100 V/cm in 1×TAE buffer.

1× TAE:

1 mM EDTA, pH = 8.0

40 mM Tris-acetate, pH = 8.0

6× DNA loading buffer:

0.025% (w/v) bromophenol blue

40% (w/v) sucrose

2.3.7. Reverse transcription reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) is an enzymatic reaction during which reverse transcriptase (RT) generates cDNA complementary to RNA. In order to perform RT-PCR, 500 ng of rat total kidney RNA was combined with autoclaved water up to 10 µl of total volume (Table 1). The reaction mixture was heated to 70 °C for 10 min, chilled on ice, and the following RT reagents were added:

Table 1. Composition of the reverse transcription reaction.

RT reaction component	Volume (µl)	Final concentration
10 mM dNTP mix	1	0.5 mM
10× RT Buffer II ($MgCl_2$ free)	2	1x
Random hexamers (50 µM)	1	2.5 µM
25 mM $MgCl_2$	4	5 mM
RNAse inhibitor (20 U/µl)	0.5	10 U
Reverse transcriptase (50 U/µl)	1	50 U

The cDNA synthesis was performed under the following conditions: the reaction mixture was incubated at 20 °C for 10 min, then at 43 °C for 75 min and at 99 °C for 5 min. The cDNA was diluted 1:3 in autoclaved water and stored either at -40 °C or used immediately.

2.3.8. Real-time polymerase chain reaction

Quantitative real-time PCR (qPCR) is used to amplify and quantify a specific DNA molecule. SYBR Green I is the most commonly used dye as a detection system. It is a double-stranded DNA intercalating dye, that fluoresces once bound to the double-stranded DNA. The amount of dye incorporated is proportional to the amount of target amplicon generated.

Reactions were performed in accordance with the manufacturer's instructions provided with a SYBR® Green PCR Kit (Table 2). All components were combined as follows:

Table 2. Composition of the real time polymerase chain reaction.

PCR reaction component	Volume (µl)	Final concentration
Platinum® Syber® Green qPCR SuperMix-UDG	13	1x
50 mM $MgCl_2$	1	2 mM
10 µM forward primer*	0.5	0.2 µM
10 µM reverse primer*	0.5	0.2 µM
cDNA template	2	not applicable
H_2O (autoclaved)	8	not applicable

Rat porphobilinogen deaminase (PBGD) served as a reference gene for qPCR reactions. The relative transcript abundance of a gene is expressed in ΔCt values ($\Delta Ct = Ct^{reference} - Ct^{target}$). The amplification of the specific PCR product was confirmed by melting curve analysis and gel electrophoresis.

The qPCR reaction was carried out as follows (Table 3):

Table 3. Programme for real time polymerase chain reaction.

Step	Time	Temperature (°C)
Activation of polymerase enzyme	2 min	50
First denaturation	5 min	95
Second denaturation*	5 s	95
Annealing*	5 s	59
Elongation*	30 s	72
Dissociation step 1	15 s	95
Dissociation step 2	1 min	60
Dissociation step 3	15 s	95
Dissociation step 4	15 s	60

*These steps were repeated for 45 cycles.

All primer sequences are listed in Table 4 in the Appendix

2.3.9. Protein isolation from tissues

Proteins were extracted from total rat kidney tissue. Kidney tissues were disrupted and ground to powder under liquid nitrogen with a mortar and pestle and homogenised by addition of tissue lysis buffer. A tissue lysate was then passed 5-8 times through a 0.9 mm gauge needle to fragment bigger clusters of tissue. Homogenised tissue was then incubated in Eppendorf tubes for 30 min on ice and centrifuged 13000 × *g* for 15 min at 4 °C. Resulting supernatant was used as tissue extracts and stored at -80 °C for further experiments.

Tissue lysis buffer:

20 mM Tris-HCl, pH = 7.5

150 mM NaCl

1 mM EDTA

1 mM EGTA

1% Trition X-100

1 mM Na_3VO_4, phosphatese inhibitor – added immediately prior to homogenisation

1 mM Complete™, protease inhibitor mix – added immediately prior to homogenisation

2.3.10. Protein quantification

Protein concentrations in tissue extracts were spectrophotometrically determined using a Fusion A153601 Reader. This protein measurement is based on the reaction between an aromatic amino acid groups and Coomassie Brilliant Blue G-250 dye. The change in colour can be spectrophotometrically measured and is proportional to the amount of proteins. A standard curve using five dilutions of bovine serum albumin (BSA) (0.05-0.4 mg/ml) was performed in parallel with every protein measurement. Sample (10 µl) was placed in a 96-well plate together with 200 µl of Bradford dye reagent. The reaction was incubated for 15 min at RT. The absorbance was then measured at 570 nm.

2.3.11. Protein gel electrophoresis

Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Before loading onto the gel, 40 µg of protein was combined with 2× SDS-loading buffer and denaturated by heating for 10 min at 95 °C. The separation of proteins was performed in the gel consisting of 5% stacking gel and 10-12% resolving gel. Electrophoresis was carried out in SDS running buffer at 120 V.

2× SDS loading buffer:

100 mM Tris-HCl, pH = 6.8
20% (v/v) glycerol
4% (w/v) SDS
200 mM dithiothreitol
0.2% (w/v) bromophenol blue

Stacking gel:

5% acrylamide:bisacrylamide
125 mM Tris-HCl, pH = 6.8
0.1% (w/v) SDS
0.1% (w/v) Ammonium persulfate (APS)
0.1% (v/v) *N,N,N',N'*-tetramethyl-ethane-1,2-diamine (TEMED)

SDS running buffer:

25 mM Tris
250 mM glycine
0.1% (w/v) SDS

Resolving gel:

10-12% acrylamide:bisacrylamide

375 mM Tris-HCl, pH = 8.8

0.1% (w/v) SDS

0.1% (w/v) APS

0.1% (v/v) TEMED

2.3.12. Protein blotting

The western blot is a technique that allows detection of specific proteins in a given sample using specific antibodies for recognition. Proteins separated by SDS-PAGE were transferred from a polyacrylamide gel to a 0.25 µm nitrocellulose membrane. Protein transfer was carried out in transfer buffer at 110 V for 1 h.

Transfer buffer (pH 7.4):

25 mM Tris

192 mM glycine

20% (v/v) methanol

2.3.13. Protein visualisation

Membranes were incubated in blocking solution for 2 h at RT followed by incubation with the appropriate primary antibodies at 4 °C overnight. Membranes were washed 5×10 min with washing buffer and then incubated with horse-radish peroxidase (HRP)-labelled secondary antibody for 1 h at RT followed by washing 5×10 min with washing buffer. Primary and secondary antibody concentrations varied depending on the antibodies used in the experiment, and are presented in Table 5. Specific bands were visualised by chemiluminescence using an Enhanced Chemiluminescence Immunoblotting system.

Blocking solution:

5% (w/v) non-fat dry milk

1× PBS

0.1% (v/v) Tween-20

1× PBST:

1× PBS

0.1% (v/v) Tween-20

2.3.14. Enzyme-linked immunosorbent assay

The quantification of rat lipocalin-2 protein in the urine of mechanically ventilated rats was carried out with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bioporto Diagnostics, Denmark) following the recommendations of the manufacturer.

2.3.15. Kidney fixation and embedding

Kidneys were extracted from rats at the end of experiments and immediately cut into three pieces in order to have every region of the organ. The middle regions were fixed by immersion for 24 h in freshly prepared 4% paraformaldehyde in 0.1 M Sorensen's phosphate buffer and then were washed for additional 24 h in 1× Sorensen's buffer at 4 °C. Specimens were kept in 50% isopropanol for 30 min followed by 3×2 h with 70% isopropanol each at 4 °C. Finally for dehydration purposes the material were put for 1 h once in 96% and twice in 100% isopropanol, respectively. After these steps kidneys were placed for 1 h in solutions of Rotihistol and isopropanol, as follows: 25% Rotihistol + 75% isopropanol, 50% Rotihistol + 50% isopropanol, 75% Rotihistol + 25% isopropanol and 100% Rotihistol. At the end of the protocol, each specimen was immersed in liquid paraffin at 60 °C overnight.

4% Paraformaldehyde in 1× Sorensen's Buffer pH=7.4

4 g paraformaldehyde

75 µl 1M NaoH

to 50 ml dH_2O

add 50 ml 2× Sorensen's Buffer

2× Sorensen's Buffer

3.48 g NaH_2PO_4

14.3 g Na_2HPO_4

and to 500 ml dH_2O

Every glass slide was silanised to protect against the detachment of cryosections and paraffin sections.

2.3.16. Paraffin sections

The H&E stain is one of the most widely used methods for diagnostic purposes to screen for pathological changes in tissue, and consists of haematoxylin (hemalum) and eosin to stain the nuclei (blue) and the cytoplasmic (red, pink, orange) fractions of cell, respectively.

The 7-μm thick sections were cut from paraffin blocks and fixed on a silanised slide. To dry the sections, every slide was placed on a slide warmer for 30 min at 38 °C followed by incubating at 55 °C overnight. The following day, the sections were dewaxed and rehydrated as follows:

- 3×10 min in xylol
- 10 min and then 3 min 100% isopropanol
- 3 min in 90%, 80%, 70%, 50% isopropanol, sequentially
- distilled water to wash

After dewaxing and rehydratation, the sections were stained as follows:

- 1.5 min in hemalum
- 2× distilled water
- 5 min in a tap water
- 30 s in eosin
- 2× distilled water
- washing in 70%, 80%, 90% isopropanol, sequentially
- 2×2 min in 100% isopropanol
- xylol until putting the mounting medium
- mounting in pertex and dry 1 h in RT

Hemalum

1 g haematoxylin
0.2 g sodium iodide
50 g aluminium potassium sulphate
fill up to 1000 ml dH_2O
Then, after 24 h:
50 g chloral hydrate
1 g citric acid monohydrate
boil and filter

Eosin

1 g Eosin G

fill up to 100 ml dH_2O

filter and before use, add 1 drop of acetic acid

2.3.17. Cryosections - immunohistochemistry

Immunohistochemistry is a method used for the visualisation and detection of antigens by antibodies in a tissue section. Different techniques can be used to visualise the antigen-antibody complex. One of the most popular is the utilisation of peroxidase coupled to an antibody. In the presence of substrate, the chemical reaction occurs, which leads to colour formation and display of the distribution of antigen. The tissue section can be then examined by microscopy.

Sections (6 μm) were cut out from kidney pieces and placed on a silanised slide. To dehydrate the sections, every slide was placed in 100% isopropanol for 10 min, and then removed from solution and left for 1 h at RT. The protocol was carried at RT on the same day as follows:

- 1× rinse in PBS
- 30 min in 1% H_2O_2 dissolved in PBS to neutralise the endogenous peroxidase activity
- 3× 2 min rinse in PBS
- 1 h incubation with primary antibody (1:500) diluted in PBS/BSA/azide solution
- 3× rinse with PBS
- 30 min incubation with secondary antibody (1:25) diluted in 5% inactivated normal rat serum and PBS
- 3× rinse with PBS
- 30 min incubation with peroxidase-anti-peroxidase (PAP) (1:100) diluted in PBS
- 3× rinse with TBS
- 10 min incubation with 3,3'-Diaminobenzidine (DAB), a substrate for peroxidase
- 3× rinse with PBS
- rinse in PBS and distilled water
- 90 s counterstaining in hemalum (diluted 1:10)
- dehydration in the row of isopropanol (50%, 70%, 80%, 90%)
- 2×2 min 100% isopropanol
- storage in xylol until moving the slide into pertex mounting medium
- mounting in pertex mounting medium and dry 1 h at RT

The titer of primary antibody was different for every antigen. On control sections only, a PBS/BSA/azide solution was used without primary antibody dilution. All solutions and apparatus which came into a contact with DAB were washed with a 12% sodium hypochlorite solution.

PBS/BSA/azide pH=7.2

0.2 g BSA

0.02 g NaN_3

made to 20 ml with 1× PBS

DAB solution

1 tablet of 3,3´-diaminobenzidine dissolved in 20 ml Tris-buffered saline

TBS, pH=7.6

6.05 g Trizma Base

9 g NaCl

HCl to adjust pH and dH_2O to a final volume of 1000 ml

2.3.18. Statistical analysis of data

Values are presented as mean ± SD, except for dot plots which present the mean. Mean values were compared using one-way ANOVA with the Newman–Keuls Multiple Comparison *post-hoc* test. The comparison was always performed between three groups with respect to time-dependence: non-ventilated, and two different time-points with the same ventilation strategy (such as non-ventilated, 10 cmH_2O PIP/2 cmH_2O PEEP 4 h and 8 h). The Grubbs' test was performed to determine whether any value from the presented group were significant outliers from the rest. Outliers were skipped, and this is always reflected in the text of the Results Section.

3. Results

The analyses presented later in this report rely critically on subtle (5 cmH_2O) increments in PIP over the range 10 cmH_2O to 20 cmH_2O. For this reason, a very careful characterisation of the haemodynamics and other parameters relevant to critical care medicine were undertaken for all three groups of ventilated rats, to facilitate the correlation of ventilation strategy with indicators of AKI.

3.1. Analysis of haemodynamics and blood gases in mechanically ventilated rats

In order to fully characterise the ventilated rat model, and to facilitate stabilisation of ventilated rats in a critical care setting, analysis of blood gases and haemodynamics was performed at different time-points. The partial pressure of oxygen in arterial blood (PaO_2) and partial pressure of carbon dioxide in arterial blood ($PaCO_2$) are good parameters describing gas exchange by the lung, and were analysed together with MAP, lactate, haemoglobin and pH. For every measurement at least 0.2 ml of blood was collected. To avoid the effects of hypovolaemia, which can occur when 10% of blood volume is lost, only five time-points were chosen (0, 60, 240, 360 and 480 min). The total rat blood volume is equal to approximately 6% body weight. All rats weighed 350-400 g, therefore, no more than 2 ml of blood was collected.

3.1.1. Measurements of blood oxygenation in mechanically ventilated rats

The blood oxygenation is measured to monitor the amount of oxygen which is dissolved in the blood. Respiratory insufficiency or inadequate management of the respiratory settings of the ventilator can lead to hypoxaemia, which later causes tissue hypoxia in distant organs, resulting in organ damage and maybe death. In this study, proper oxygenation was maintained by manipulating ventilator settings (as outlined in Section 2.3.2.), but the PIP/PEEP value was always held constant within the experimental group.

Rats ventilated for 4 h and 8 h exhibited PaO_2 values which varied depending on the ventilation strategy applied (Figure 5). After 60 min, a drop in the PaO_2 values was observed in the 10 cmH_2O PIP/2 cmH_2O PEEP group, from 140 mmHg, reaching a plateau level of 80 mmHg by 8 h. This decrease suggests that these animals were under-ventilated.

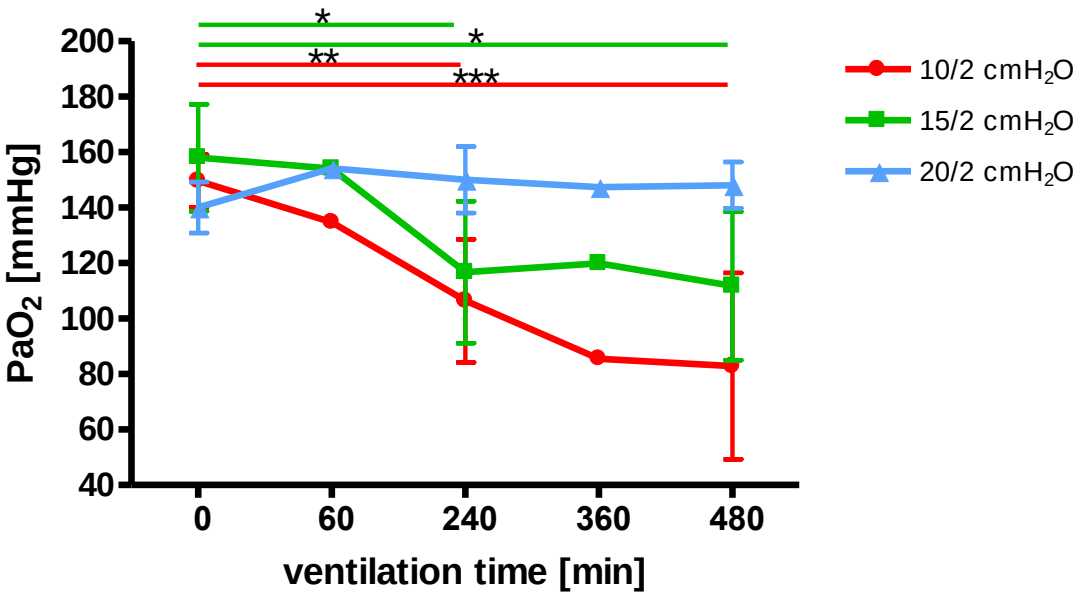

Figure 5. Blood oxygenation of ventilated rats. Blood oxygenation (reflected by PaO_2) of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). In the interests of clarity, error bars have been omitted for the intermediate time-points. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

A drop was also observed in the PaO_2 level in the 15 cmH_2O PIP/2 cmH_2O PEEP group after 60 min. For the first hour of the experiment the PaO_2 value stabilised at 150 mmHg, after which the PaO_2 decreased to 120 mmHg by 4 h, after which the PaO_2 remained stable until the end of experiment.

The 20 cmH_2O PIP/2 cmH_2O PEEP group exhibited a stable PaO_2 of 150 mmHg over the entire time-course of experiment. Subsequent analyses of $PaCO_2$ levels revealed that this group of rats was probably over-ventilated, as discussed in the following section (Section 3.1.2.).

Based on PaO_2 data, animals ventilated with a 15 cmH_2O PIP/2 cmH_2O PEEP strategy seemed to be optimally ventilated, whereas animals in the other two groups (10 cmH_2O PIP/2 cmH_2O PEEP and 20 cmH_2O PIP/2 cmH_2O PEEP) appeared to be under- and over- ventilated, respectively.

3.1.2. Measurement of blood CO_2 in mechanically ventilated rats

The CO_2 functions as a regulator of breathing. The level of arterial CO_2 is usually maintained at approximately 40 mmHg. When this value is altered, it indicates a respiratory problem or a metabolic issue, which then requires normalisation of blood pH. It is assumed that hypoventilation occurs when high $PaCO_2$ are detected during

blood gas measurements. Similarly, low $PaCO_2$ levels indicate over-ventilation. High $PaCO_2$ levels can lead to respiratory acidosis, while low $PaCO_2$ levels provoke respiratory alkalosis, both of which are life-threatening disease states.

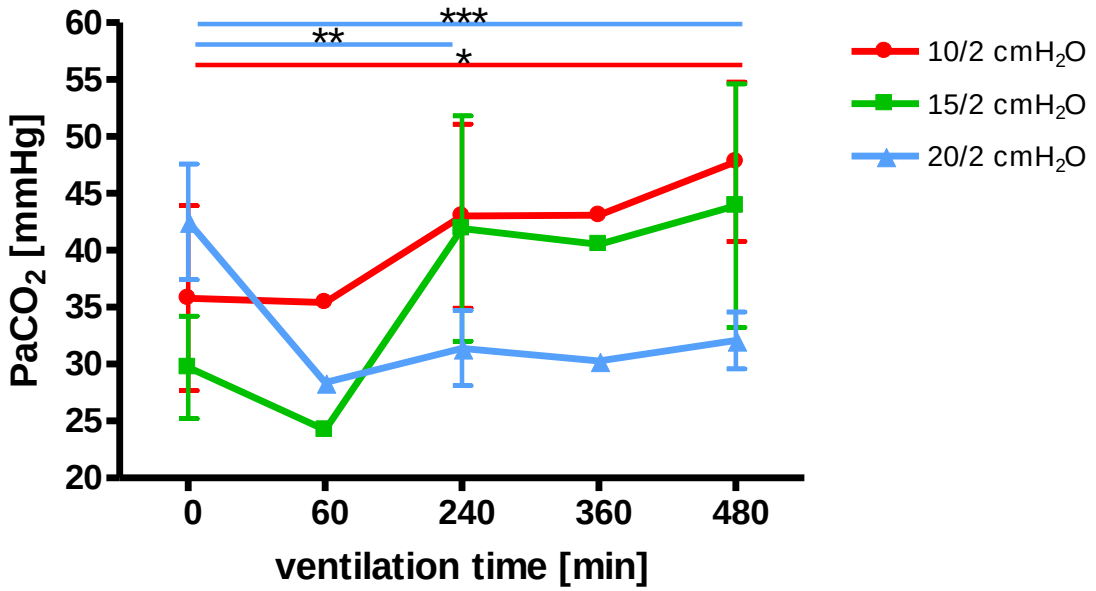

Figure 6. Blood $PaCO_2$ of ventilated rats. The $PaCO_2$ level in the blood of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). In the interests of clarity, error bars have been omitted for the intermediate time-points. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Rats ventilated with 15 cmH_2O PIP/2 cmH_2O PEEP did not maintain stable $PaCO_2$ levels (Figure 6). It was observed that at early time-points, the $PaCO_2$ values seemed to be below physiological levels (hypocapnia), but at late time-points, values returned to 40 mmHg (normocapnia). It can be assumed that the 15 cmH_2O PIP/ 2 cmH_2O PEEP group was properly ventilated with respect to $PaCO_2$ levels at the later time-points.

Over the 8-h ventilation time-course, the 10 cmH_2O PIP/2 cmH_2O PEEP group tended to have increased $PaCO_2$ levels, reaching 48 mmHg at 8 h, suggesting under-ventilation. A completely opposite effect was seen for the 20 cmH_2O PIP/2 cmH_2O PEEP group, which appeared to be over-ventilated, with a $PaCO_2$ level of about 42 mmHg at the beginning of the time-course and about 32 mmHg at the end of the time-course. Overall, these results are in agreement with the previous observations regarding the PaO_2 values, indicating that animals ventilated with a 15 cmH_2O PIP/ 2 cmH_2O PEEP strategy were optimally ventilated, whereas the 10 cmH_2O PIP/ 2 cmH_2O PEEP group was under-ventilated and 20 cmH_2O PIP/2 cmH_2O PEEP group was over-ventilated.

3.1.3. Blood pH values in mechanically ventilated rats

Acid-base homeostasis is a critical issue in an intensive-care setting and is normally tightly regulated. The normal range of pH is required for effective enzyme activity and proper protein structure. The pH value is related to blood CO_2 level. A powerful physiological mechanism exists to maintain pH in the normal physiological range which is 7.35–7.45. Both respiratory insufficiency and metabolic issues impact pH. If the blood pH decreases, the body will compensate by expelling more CO_2. The kidneys are also able to regulate the pH level by intensifying the metabolism of bicarbonate and hydrogen.

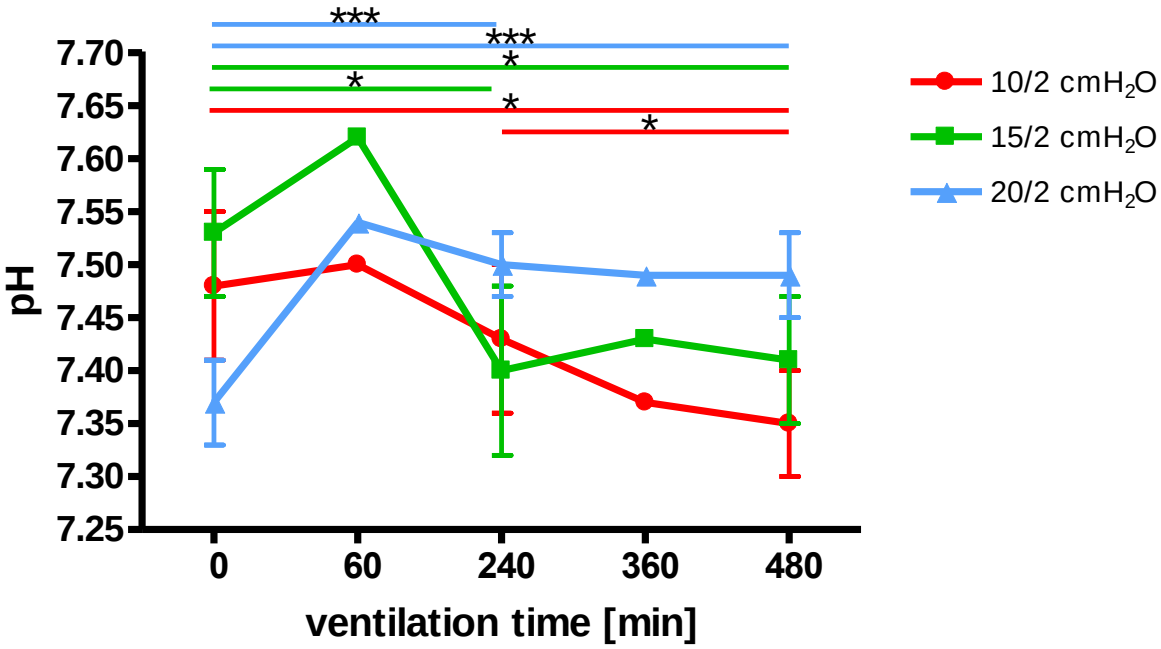

Figure 7. Blood pH of ventilated rats. Blood pH of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). In the interests of clarity, error bars have been omitted for the intermediate time-points. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

As illustrated in Figure 7, over the course of an 8-h ventilation period, it was observed that the 10 cmH_2O PIP/2 cmH_2O PEEP group tended to have a decreased blood pH with a corresponding increase in blood $PaCO_2$ levels. There was a drop in pH from 7.47 at 60 min to 7.35 at 8 h.

The 20 cmH_2O PIP/2 cmH_2O PEEP group exhibited increased values of pH, which were already elevated by 60 min of mechanical ventilation, from pH 7.36 at the zero time-point, to 7.49 at 60 min, and remained at that level for the remainder of the experiment.

The 15 cmH_2O PIP/2 cmH_2O PEEP group did not maintain stable pH. The pH increased after 60 min to 7.62, compared to the zero time-point (7.53) and then dropped to physiological levels (7.41).

Animals that were thought to be under-ventilated (the 10 cmH_2O PIP/2 cmH_2O PEEP group) exhibited high $PaCO_2$ values and lower pH, indicating a trend towards the development of acidosis. The opposite situation was observed in over-ventilated animals (the 20 cmH_2O PIP/2 cmH_2O PEEP group), where low $PaCO_2$ and high pH values were observed, suggesting respiratory alkalosis in these animals. The most stable animals, in terms of blood pH, appeared to be those in the 15 cmH_2O PIP/ 2 cmH_2O PEEP group.

3.1.4. Mean arterial pressure in mechanically ventilated rats

Mean arterial pressure is defined by the mean pressure of the blood in the arteries throughout the cardiac cycle, and is determined by cardiac output, systemic vascular resistance and central venous pressure. The MAP is frequently used to assess the haemodynamic status of patient. Since cardiac output is affected by mechanical ventilation, it is very important to monitor MAP. When the MAP value drops, this can lead to reduced perfusion of the organs. In this case, the blood flow in the end organ will be decreased and ischaemic injury can occur. It is suggested to always maintain the MAP above 60 mmHg to sustain proper organ perfusion.

The MAP values, for every group of animals over the 8-h time-course of mechanical ventilation, were maintained at an appropriate level, suggesting proper organ perfusion (Figure 8). The MAP for the 10 cmH_2O PIP/2 cmH_2O PEEP and 20 cmH_2O PIP/2 cmH_2O PEEP groups was constant over the course of the experiment, while the MAP for the 15 cmH_2O PIP/2 cmH_2O PEEP group increased between 0 to 240 min over the time-course of the experiment, reaching 110 mmHg, and then returned to 85 mmHg. A MAP of about 80 mmHg was observed for animals ventilated with 15 cmH_2O PIP/2 cmH_2O PEEP and 20 cmH_2O PIP/2 cmH_2O PEEP at the end of experiment. The animals ventilated with the 10 cmH_2O PIP/2 cmH_2O PEEP exhibited an increased MAP of approximately 100-110 mmHg, which could result from decreased PaO_2, where lower oxygenation is compensated for by increased cardiac output.

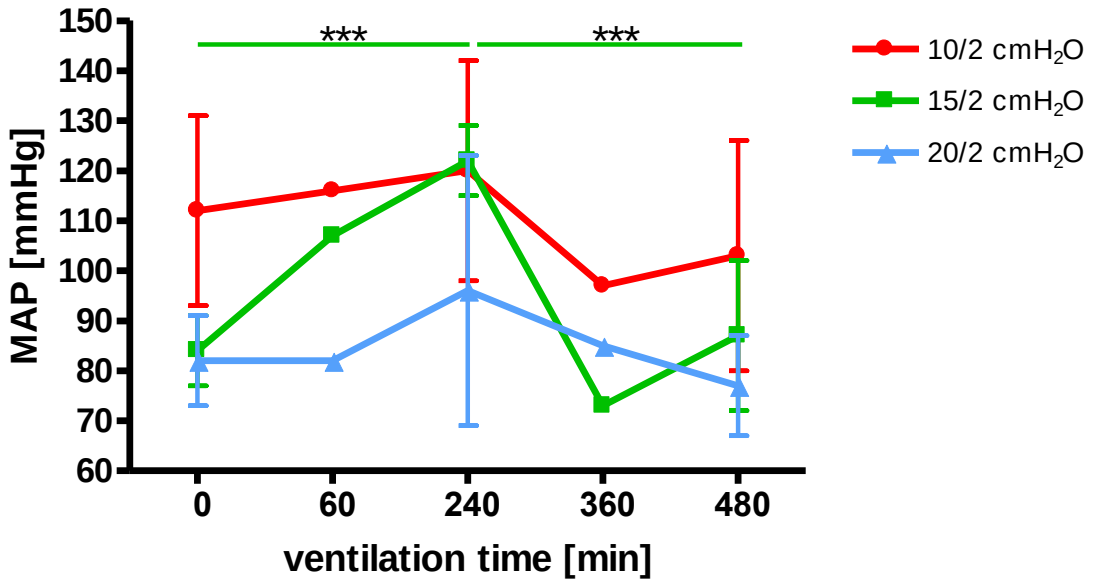

Figure 8. Mean arterial pressure of ventilated rats. Mean arterial pressure (MAP) of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). In the interests of clarity, error bars have been omitted for the intermediate time-points. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.1.5. Lactate values in mechanically ventilated rats

Lactate is a hydroxyl carboxylic acid, an organic component produced in the muscle during excessive exercise. Under normal physiological conditions, the production of lactate is compensated for by removal by tissues. This is not the case under anaerobic conditions, where the production of lactate is faster than lactate utilisation. The concentration of lactate in the blood is usually 0.7–1.8 mM under normal physiological conditions, but can increase by up to 10 times these levels during power exercises. Acidosis associated with prolonged elevation of lactate during anaerobic conditions is one of the factors contributing to loss of homeostasis. Lactate serves as an important biomarker of anaerobic metabolism in critical care setting.

Surprisingly, the most physiological values of lactate were observed for the 10 cmH_2O PIP/2 cmH_2O PEEP ventilated group, where lactate levels were between 1.8 to 0.7 mM over the course of ventilation (Figure 9). Other ventilation strategies (15 cmH_2O PIP/2 cmH_2O PEEP and 20 cmH_2O PIP/2 cmH_2O PEEP) seemed to generate elevated levels of blood lactate after 60 min of ventilation, reaching 2.9 mM for the 20 cmH_2O PIP/2 cmH_2O PEEP group and 2.4 mM for the 15 cmH_2O PIP/ 2 cmH_2O PEEP group, suggesting anaerobic processes taking place at the beginning

of the experiment. At later time-points for these two groups, the lactate values dropped to 0.8-1 mM, which are within the normal physiological range. It seems that the first 60 min of mechanical ventilation was the time of adaptation to ventilation and the conditions applied with the ventilator settings. After that time, lactate returned to physiological conditions, suggesting that blood lactate levels did not play any role in the loss of homeostasis with these experimental settings.

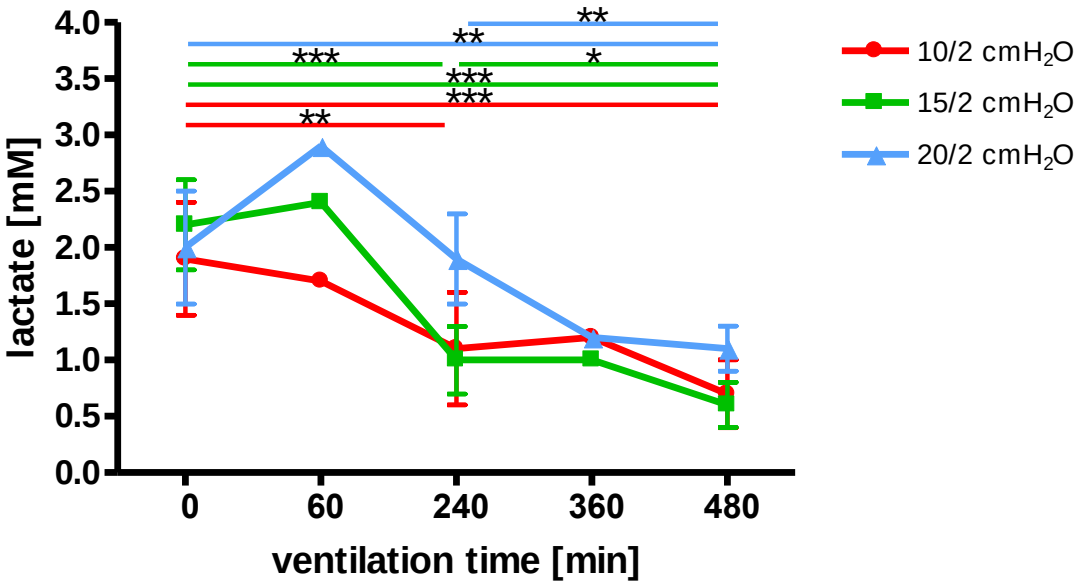

Figure 9. Blood lactate of ventilated rats. Blood lactate values of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). In the interests of clarity, error bars have been omitted for the intermediate time-points. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.1.6. Haemoglobin content in mechanically ventilated rats

Haemoglobin is a metalloprotein that carries the oxygen and CO_2 (15% of total transport) between the lungs and extrapulmonary organs, and is present mainly in red blood cells. In other cells, such as macrophages and mesangila cells, haemoglobin plays a role as a anti-oxidant or iron regulator. Haemoglobin deficiency leads to an abrogated capacity for oxygen transport and tissue delivery, which can cause hypoxia in organs. Common causes of haemoglobin deficiency include anaemia, chemotherapy, kidney failure and genetic disorders.

As illustrated in Figure 10, haemoglobin values over the 8-h time-course of mechanical ventilation varied with time and ventilator settings. Surprisingly, the starting value for the 20 cmH_2O PIP/2 cmH_2O PEEP group, which was 7.0 mM, differed from

the other two ventilation groups, which had haemoglobin levels of 8.0 mM. After 4 h of mechanical ventilation, all groups reached the same level of circulating haemoglobin of 8.5 mM. During the last 4 h of the experimental time-course, all groups tended to have decreased levels of heamoglobin, compared to the 4 h time-point, and all varied from 7.0 to 8.0 mM. Perhaps loss of blood due to blood collection for biochemical and blood gas analyses, or metabolic stress related to energy use, and limiting nutrition; led to variability in haemoglobin levels.

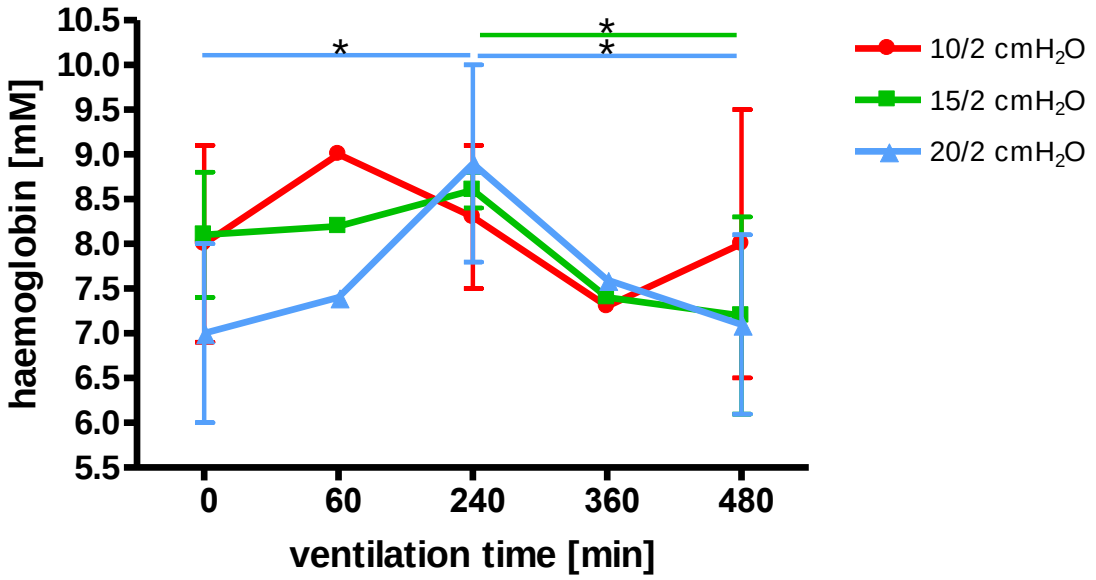

Figure 10. Haemoglobin content of ventilated rats. Haemoglobin content of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). In the interests of clarity, error bars have been omitted for the intermediate time-points. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.2. Serum creatinine levels in mechanically ventilated rats

Renal function can be assessed by measuring serum creatinine. This is a useful and inexpensive indicator of kidney function, but also has many disadvantages. Creatinine is continuously produced by skeletal muscle cells where it is a non-protein break-down product of creatine phosphate. Under normal conditions, creatinine is freely filtered by the kidney glomerulus, and secreted by proximal tubules. Serum creatinine rises in the case of renal dysfunction, when the filtration process is diminished. Additionally, increased serum levels of creatinine are observed in urinary tract obstruction, muscle diseases, congestive heart failure and shock. The reference values for serum creatinine vary from approximately 0.8-1.4 mg/dl for males, and

0.6-1.1 mg/dl for females. Serum creatinine levels can also be affected by pregnancy, age, muscle mass and inadequate diet. Important to add is that the serum creatinine level is not increased until at least half of the nephrons of the kidney are damaged. Increases in serum creatinine levels of at least 50% over the baseline can indicate AKI. For this reason, serum creatinine is regarded rather as a marker of late kidney injury.

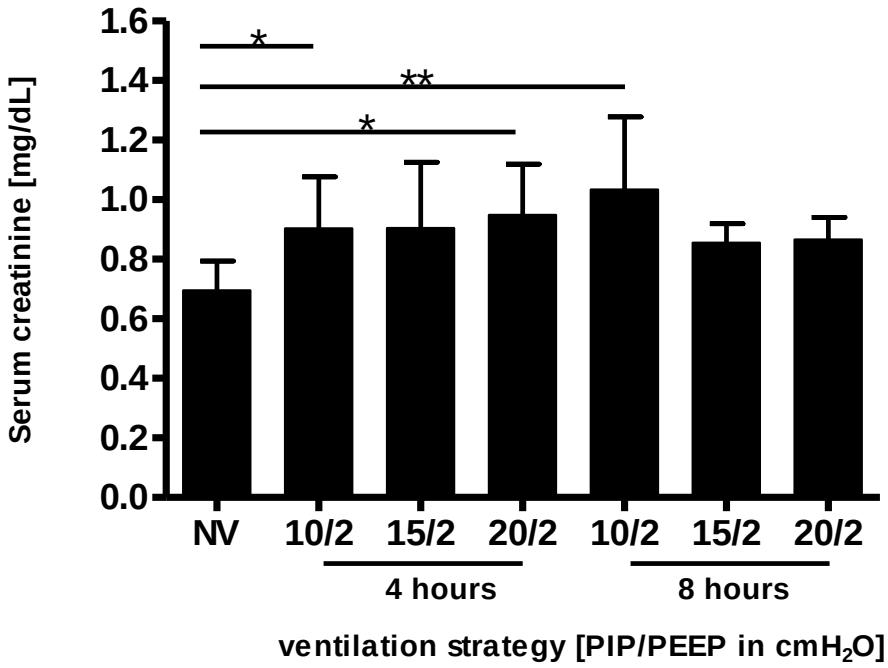

Figure 11. Serum creatinine of ventilated rats. Serum creatinine levels of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, p<0.05; **, p<0.01; ***, p<0.001.

Serum creatinine values were elevated in all mechanically ventilated animals comparing to non-ventilated animals (Figure 11). The only differences in the observations were seen when comparing non-ventilated animals with 20 cmH_2O PIP/ 2 cmH_2O PEEP group at 4-h time-point or 4 h and 8 h of mechanical ventilation in the 10 cmH_2O PIP/2 cmH_2O PEEP. Additionally, for all experimental groups, serum creatinine levels did not exceed 50% over baseline values in control animals, and were thus, within the physiological range.

3.3. Total protein level of BAL fluid in mechanically ventilated rats

Bronchoalveolar lavage is a diagnostic procedure and is routinely performed to diagnose and control lung diseases, in particular, some lung cancers, pneumonia, immune system problems and ALI/ARDS. Collection of the BALF allows determination of the composition of epithelial lining fluid in the lung and the protein content of fluid in the airways. Total protein in BALF will increase in the case of capillary leakage and alveolar disruption, which is one of the hallmarks of ALI/ARDS. This diagnostic method was needed to test whether, in this study, any of the ventilation strategies caused lung injury, and thus, increased BALF protein.

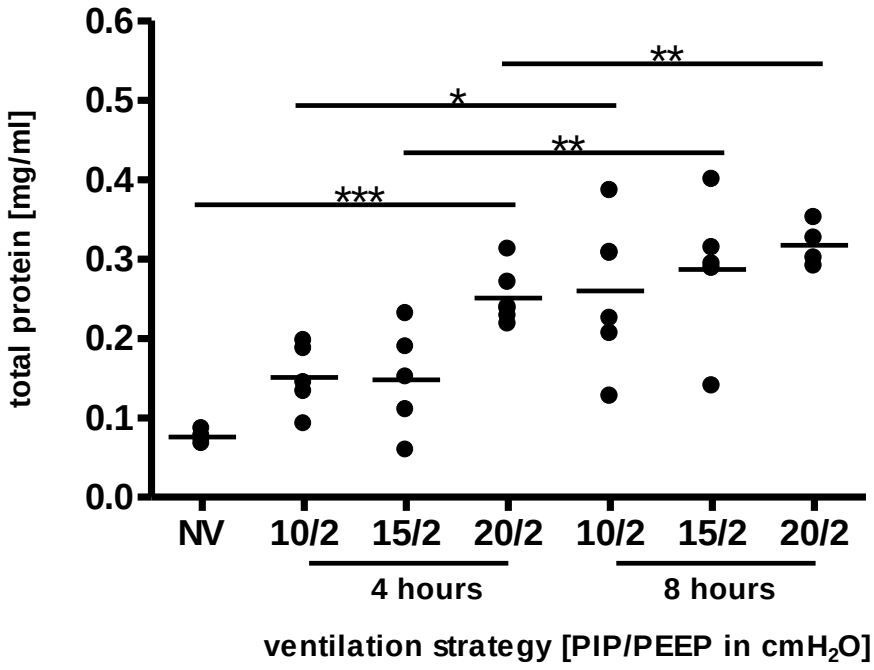

Figure 12. The BALF protein of ventilated rats. The BALF protein (Bradford assay) of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

The total protein level in BALF increased significantly in all experimental groups after 8 h of mechanical ventilation (statistics not indicated on the graph). However, only the 20 cmH_2O PIP/2 cmH_2O PEEP group exhibited significant difference at the 4-h time-point compared with non-ventilated controls. Interestingly, all animals ventilated for 8 h exhibited an increase in BALF protein compared to the 4-h ventilation group (Figure 12). Ventilation at 20 cmH_2O PIP/2 cmH_2O PEEP for 8 h generated more lung damage than did 20 cmH_2O PIP/2 cmH_2O PEEP group for 4 h. This was also the case

for the 10 cmH_2O PIP/2 cmH_2O PEEP and 15 cmH_2O PIP/2 cmH_2O PEEP groups comparing these two time-points.

These observations demonstrate that mechanical ventilation of rats with a 20 cmH_2O PIP/2 cmH_2O PEEP ventilation strategy could provoke lung injury within 4 h. Furthermore, all ventilation strategies provoked lung injury comparing the 8-h with the 4-h time-points, using increased BALF protein levels as a marker of lung injury.

3.4. Kidney expression of lipocalin-2 mRNA in mechanically ventilated rats

Lipocalin-2 is regarded as a most promising biomarker of AKI. Studies on the mRNA levels of kidney tissue reveal changes in gene expression and are usually the first step to investigate the power of a potential biomarker as a diagnostic tool. Further steps include analyses at the protein level in the tissue or body fluids.

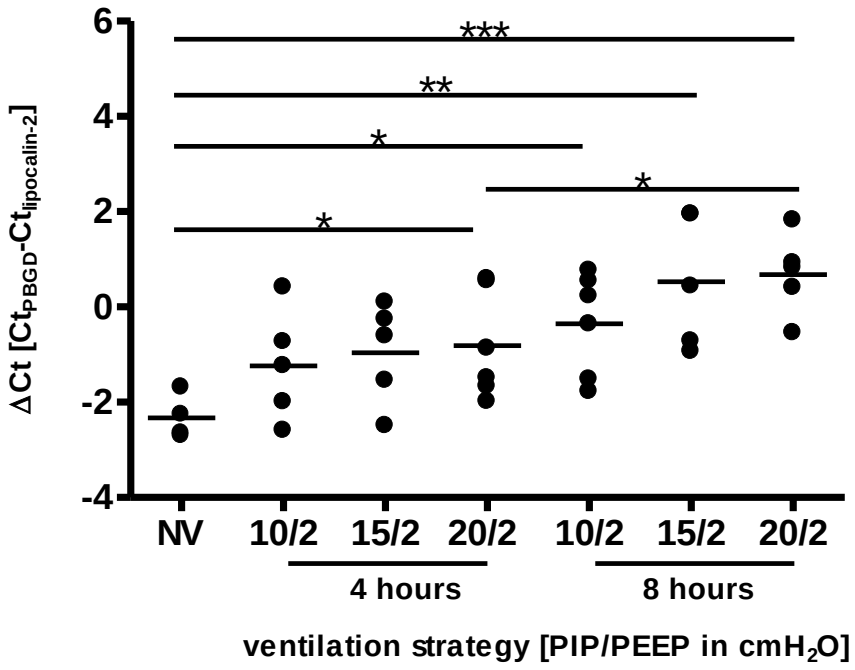

Figure 13. Kidney mRNA expression of lipocalin-2. The mRNA expression of kidney lipocalin-2 of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). Quantitative reverse transcriptase PCR was performed with PBGD serving as a reference gene. No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman-Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

As illustrated in Figure 13, lipocalin-2 expression was changed when different ventilation strategies were applied. No significant increases in the mRNA expression levels of lipocalin-2 were detected between 4 h and 8 h of mechanical ventilation within

the 10 cmH_2O PIP/2 cmH_2O PEEP and 15 cmH_2O PIP/2 cmH_2O PEEP groups, however, pronounced changes were already evident in the 20 cmH_2O PIP/2 cmH_2O PEEP 8-h group compared to the corresponding 4-h group. Interestingly, all groups manifested changes at 8 h compared to non-ventilated animals. In conclusion, lipocalin-2 levels were up-regulated in a time-dependent manner within all ventilation strategies applied. However, the 10 cmH_2O PIP/2 cmH_2O PEEP group and the 15 cmH_2O PIP/2 cmH_2O PEEP group exhibited pronounced elevations in lipocalin-2 gene expression after 8 h, whereas the 20 cmH_2O PIP/2 cmH_2O PEEP group exhibited the gene expression changes as early as 4 h, suggesting that this ventilation strategy accelerated the up-regulation of lipocalin-2 expression in the kidney.

3.5. Kidney expression of Kim1 mRNA in mechanically ventilated rats

Kidney injury molecule 1 together with lipocalin-2, is under evaluation as a marker of AKI. Importantly, Kim1 is reported to be detected later than lipocalin-2 in AKI, but the expression of Kim1 is less affected by inflammatory processes. Nevertheless the combination of these two markers might be a useful diagnostic tool for AKI.

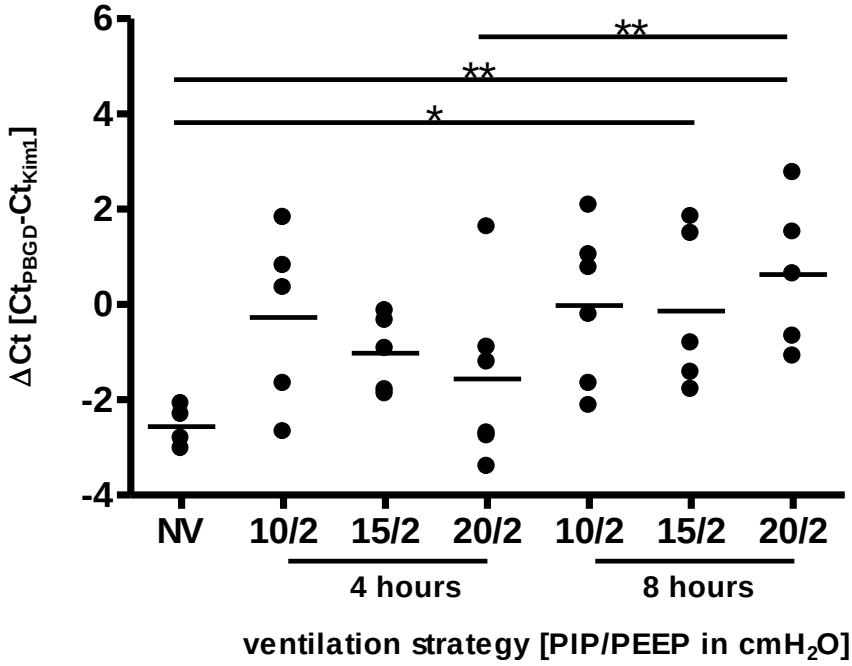

Figure 14. Kidney mRNA expression of Kim1. The kidney mRNA expression of Kim1 of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). Quantitative reverse transcriptase PCR was performed with PBGD serving as a reference gene. No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, p<0.05; **, p<0.01; ***, p<0.001.

The 20 cmH_2O PIP/2 cmH_2O PEEP group exhibited significantly increased kidney mRNA expression levels of Kim1 between 4 h and 8 h of mechanical ventilation, which was not observed in the other two ventilation strategies (Figure 14). Additionally, pronounced changes were seen at the 8-h time-point in the 15 cmH_2O PIP/2 cmH_2O PEEP and 20 cmH_2O PIP/2 cmH_2O PEEP groups but not in the 10 cmH_2O PIP/ 2 cmH_2O PEEP group *versus* non-ventilated animals. Thus, the 15 cmH_2O PIP/ 2 cmH_2O PEEP and 20 cmH_2O PIP/2 cmH_2O PEEP exhibited some damage to the kidneys in regard of Kim1 as a biomarker of AKI. Comparing lipocalin-2 and Kim1, both genes exhibited time-dependent changes in mRNA expression in the kidney of mechanically ventilated rats. Additionally, lipocalin-2 expression was up-regulated earlier in comparison with Kim1 in the evolution of AKI in this model.

3.6. Kidney expression of Hmox1 mRNA in mechanically ventilated rats

Haeme oxygenase 1 (Hmox1) is one of the three isoforms of the haeme oxygenase gene, and responds to different stimuli including hypoxia, oxidative stress and inflammation. Interestingly, Hmox1 has a very potent anti-inflammatory, anti-apoptotic and anti-oxidative properties and is activated as a factor responsible for maintaining tissue homeostasis (Slebos, Ryter and Choi 2003). Moreover, Hmox1 is a potent modulator of the immune response (such as limiting leukocyte recruitment and activation) and platelet aggregation (Ferenbach, Kluth and Hughes 2010). Changes in Hmox1 expression in the kidney may reflect the possible mechanism by which the kidneys are affected and damaged during mechanical ventilation.

No changes were observed in Hmox1 gene expression between 4 h and 8 h in the 15 cmH_2O PIP/2 cmH_2O PEEP and 20 cmH_2O PIP/2 cmH_2O PEEP groups (Figure 15). This was not the case for the 10 cmH_2O PIP/2 cmH_2O PEEP group, where changes were observed comparing the 4-h time-point with the corresponding 8-h time-point, and with non-ventilated animals. Interestingly, after 4 h mechanical ventilation, the 10 cmH_2O PIP/2 cmH_2O PEEP group exhibited increased expression of the Hmox1 gene, while at the end of experiment, gene expression returned to basal levels. No differences were observed in Hmox1 gene expression in the 15 cmH_2O PIP/2 cmH_2O PEEP group compared to non-ventilated animals but Hmox1 tended to be increased after 4 h of experiments. The 20 cmH_2O PIP/2 cmH_2O PEEP group displayed increased Hmox1 gene expression after 4 h and 8 h of mechanical ventilation compared to non-ventilated animals (one outlier was found when the Grubbs' test was applied).

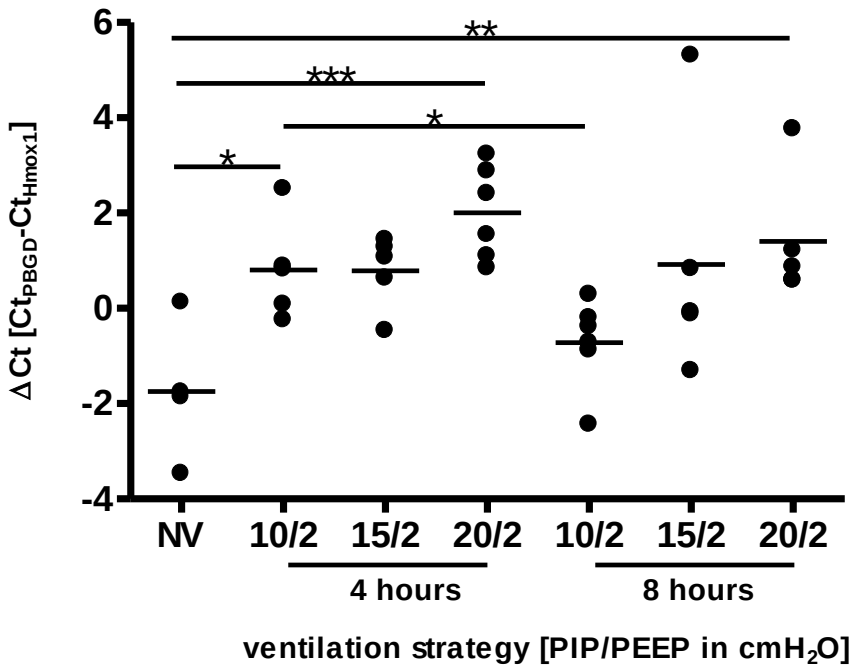

Figure 15. Kidney mRNA expression of Hmox1. The kidney mRNA expression of Hmox1 of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). Quantitative reverse transcriptase PCR was performed with PBGD serving as a reference gene. No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

In this study, the mechanism by which Hmox1 gene expression was altered in the kidneys of mechanically ventilated rats is difficult to define. However, an inflammatory response is one of the first factors triggered by mechanical ventilation. All groups tended to have elevated levels of Hmox1 mRNA after 4 h of mechanical ventilation. However, only the 20 cmH_2O PIP/2 cmH_2O PEEP group exhibited an increase in Hmox1 gene expression after 8 h of mechanical ventilation, while for the other two ventilation strategies, Hmox1 gene expression returned to basal levels. The effect of mechanical ventilation on Hmox1 gene expression in the 20 cmH_2O PIP/ 2 cmH_2O PEEP group appeared to be maintained over the course of the experiment, while for 10 cmH_2O PIP/2 cmH_2O PEEP and 15 cmH_2O PIP/2 cmH_2O PEEP Hmox1 mRNA expression fluctuated: peaking at 4 h mechanical ventilation, and returning to basal levels by 8 h.

The analysis of blood gases revealed that the 20 cmH_2O PIP/2 cmH_2O PEEP group was over-ventilated (Figure 5) and also exhibited increased Hmox1 levels over the time-course of the experiment compared to other mechanically ventilated groups.

Therefore, the 20 cmH_2O PIP/2 cmH_2O PEEP could favour the generation of ROS which might contribute to tissue injury.

3.7. Urinary lipocalin-2 adjusted to urinary creatinine in mechanically ventilated rats

Potential biomarkers of kidney diseases should be easily detected in the urine or serum. The urine is a waste product secreted by functioning kidneys and can be used in diagnostic tests to investigate biomarker levels. When the kidneys are damaged, the levels of biomarkers can be correlated with the standard values estimated for healthy individuals. Lipocalin-2 is believed to be elevated in the urine during the onset of AKI (Shemin and Dworkin 2011, Singer et al. 2011).

Since the values of creatinine in the urine are variable, the levels of urinary biomarkers are more meaningful after adjustment its value to the urinary creatinine level. In this case, urinary creatinine serves as a reference substance. One of the major factors influencing the urinary creatinine level is dehydration which leads to increased values of this waste product in the urine, particularily during prolonged experimental settings.

As illustrated in Figure 16, after adjustment of urinary lipocalin-2 values to urinary creatinine levels, no differences were observed for the 10 cmH_2O PIP/ 2 cmH_2O PEEP group comparing the 4-h or the 8-h time-points, and non-ventilated animals. It is likely that due to the high inter-animal variability within the group, the magnitude of the error did not yield mean values that were statistically significantly different from one another. Interestingly, the 20 cmH_2O PIP/2 cmH_2O PEEP group exhibited increased values of urinary lipocalin-2 values adjusted to urinary creatinine levels when comparing the 8-h group with corresponding 4-h and non-ventilated groups. Both the 10 cmH_2O PIP/2 cmH_2O PEEP and 20 cmH_2O PIP/2 cmH_2O PEEP groups contained one outlier (analysed by Grubbs' test) in the values obtained from urinary lipocalin-2/creatinine ratio. The outliers were omitted and then statistical analyses were performed on both groups.

Surprisingly, the 15 cmH_2O PIP/2 cmH_2O PEEP group also exhibited an increase in the urinary lipocalin-2/creatinine ratio at 4 h and 8 h of mechanical ventilation compared to non-ventilated animals. There was only a small variability in the value of urinary lipocalin-2/creatinine ratio in this group which could explain statistical significance in this experimental group.

All experimental groups tended to have increased lipocalin-2/creatinine levels in the urine of animals ventilated for 8 h compared to non-ventilated animals. It is not known what the reference urinary lipocalin-2/creatinine value is, which allows discriminating between AKI and non-AKI individuals, therefore, it is not suitable to judge at that moment whether any experimental group developed AKI.

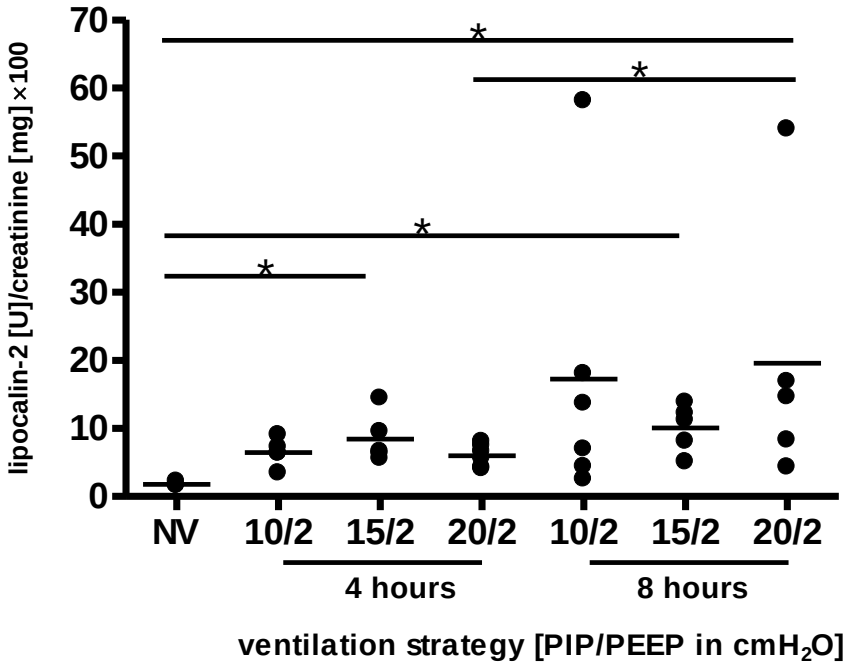

Figure 16. Urinary lipocalin-2/creatinine ratio. The lipocalin-2/creatinine ratio measured in urine of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). Enzyme-linked immunosorbent assay wa performed to measure the amount of urinary lipocalin-2 in ventilated rats. No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.8. Expression of kidney Cxcl1 mRNA in mechanically ventilated rats

Chemokine (C-X-C motif) ligand 1 (Cxcl1) is a one of the major chemokines released by the cells which activate inflammatory processes, and together with chemokine (C-X-C motif) ligand 8 (Cxcl8), is one of the most potent chemokines that recruits neutrophils, which results in modulation of the immune response. Cxcl1 is expressed on monocytes, epithelial cells, and neutrophils. During the evolution of AKI, Cxcl1 levels are up-regulated, and serve as potent attractants to immune cells to the site of inflammation. Usually, this elevation of Cxcl1 levels is transient and decreases

when the stimuli, and recruitment of the cells to the kidney is halted or inflammatory processes need to be diminished.

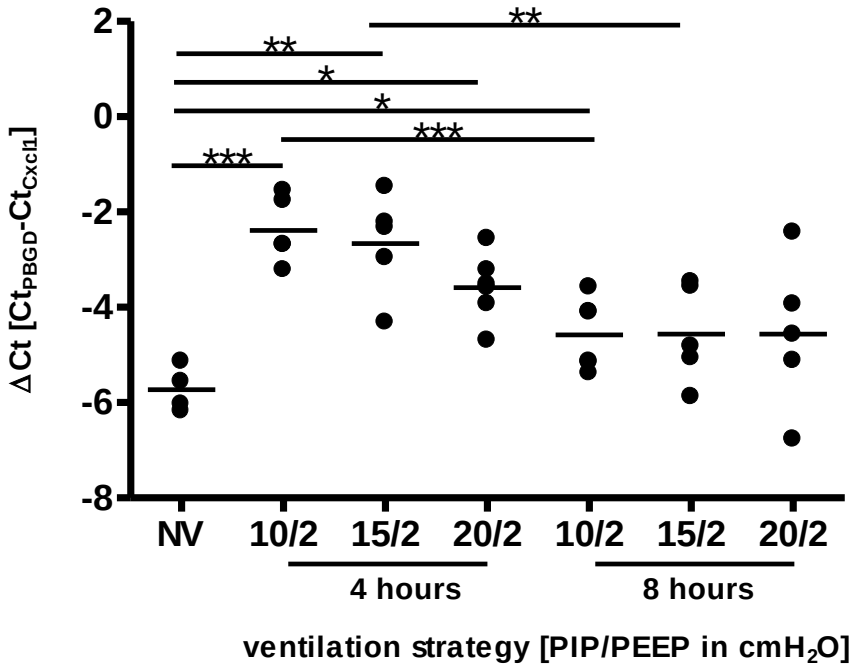

Figure 17. Kidney mRNA expression of Cxcl1. The kidney Cxcl1 mRNA expression of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). Quantitative reverse transcriptase PCR was performed with PBGD serving as a reference gene. No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, p<0.05; **, p<0.01; ***, p<0.001.

In general, kidney Cxcl1 expression was elevated in all groups at 4 h, and returned to baseline levels by 8 h (Figure 17). This might be interpreted to mean that mechanical ventilation stimulates the kidney to release chemoattractants such as Cxcl1, a potent neutrophil chemokine.

3.9. Expression of kidney Ccl2 mRNA in mechanically ventilated rats

Chemokine (C-C motif) ligand 2 (Ccl2) is regarded as a one of the most potent chemokines that recruits monocytes (primarily macrophages) to the site of tissue injury and plays a central role during the onset of AKI. Thus, as described for Cxcl1, a neutrophil chemoattractant, in Section 3.8; Ccl2 expression changes may suggest macrophage recruitment by the kidney in response to mechanical ventilation.

The mRNA expression of Ccl2 was affected by mechanical ventilation in the 10 cmH_2O PIP/2 cmH_2O PEEP group when comparing 8-h time-point with 4-h time-

point and non-ventilated animals (Figure 18). The 8-h group exhibited a decrease in Ccl2 mRNA levels that were below basal mRNA levels observed in non-ventilated animals. The other two groups (15 cmH_2O PIP/2 cmH_2O PEEP and 20 cmH_2O PIP/ 2 cmH_2O PEEP) tended to exhibit the same trend as the 10 cmH_2O PIP/2 cmH_2O PEEP group, but the observed changes were not significant.

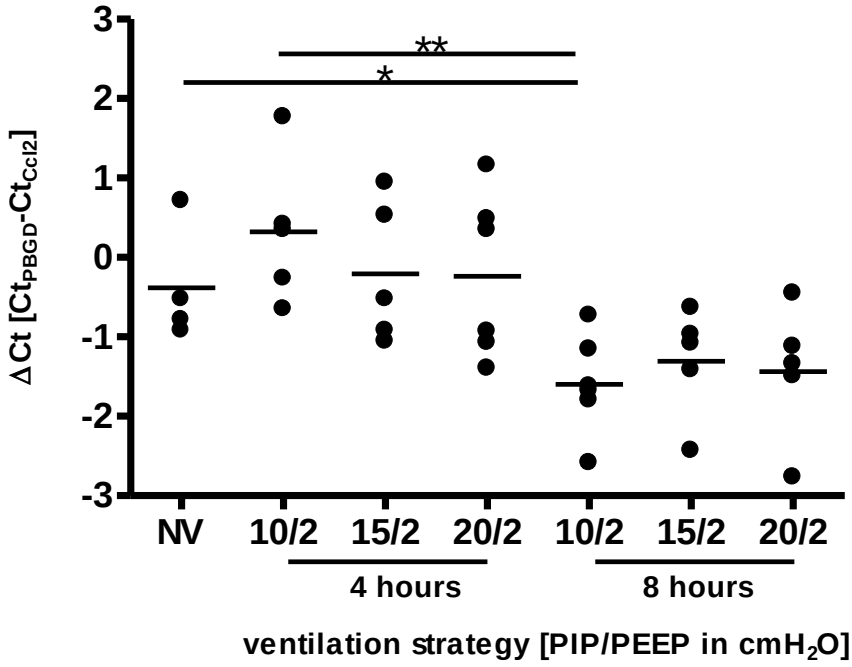

Figure 18. Kidney mRNA expression of Ccl2. The kidney Ccl2 mRNA expression of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory presure (PEEP) (2 cmH_2O). Quantitative reverse transcriptase PCR was performed with PBGD serving as a reference gene. No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, p<0.05; **, p<0.01; ***, p<0.001.

The decrease in Ccl2 mRNA levels at the 8-h time-point may reflect the engagement of anti-inflammatory pathways (for example, the early induction of Cxcl1 at 4 h, which was decreased by 8 h; Section 3.8). The recruited monocytes probably limited their own accumulation by blocking and down-regulating the expression of Ccl2, which was observed comparing the 8-h group with non-ventilated animals.

3.10. Kidney lipocalin-2 and Kim1 protein expression in mechanically ventilated rats

Usually mRNA levels reflect changes that should also be visible at the protein level. A western blot was performed in order to check whether there was a correlation between increased levels of lipocalin-2 and Kim1 mRNA in the kidney with

corresponding protein levels in the tissue. It is known that differences in the degradation, processing, storage, variety of splice variants and protein post-translational modifications (such as glycosylation, phosphorylation) disturb the balance between mRNA and a protein expression.

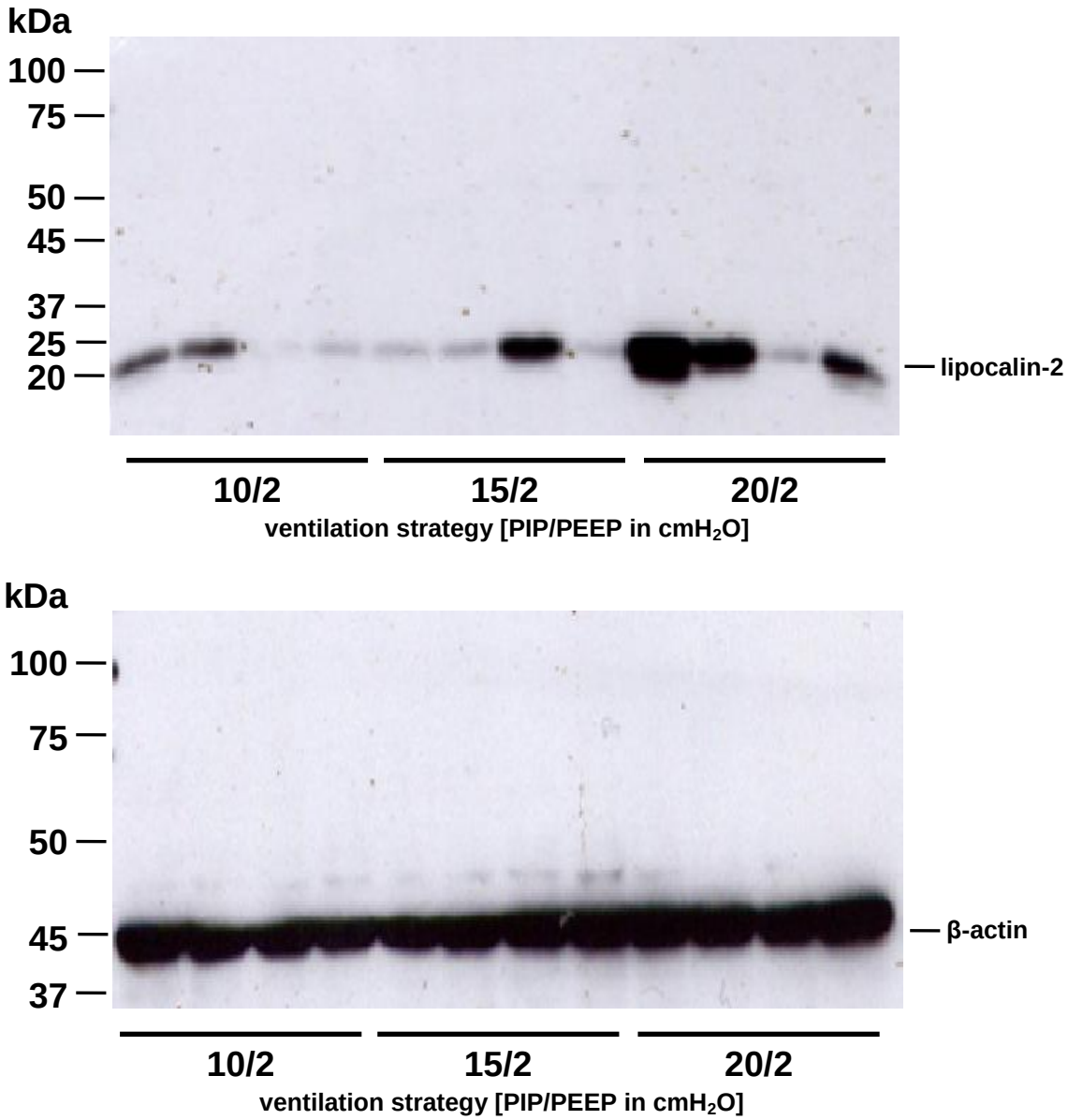

Figure 19. Kidney protein expression of lipocalin-2. The protein expression of lipocalin-2 (22 kDa) in kidney of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O) for 8 h. β-actin (45 kDa) served as a reference protein.

For lipocalin-2, one specific band was detected at approximately 25 kDa, while many bands were observed for Kim1 because this protein is known to be variably glycosylated, with molecular masses between 50 kDa and 80 kDa. For lipocalin-2, as well as for Kim1, the most pronounced protein expression was seen for the

20 cmH_2O PIP/2 cmH_2O PEEP group after 8 h (Figure 19 and Figure 20). This was not only the case when comparing different ventilation strategies within the 8-h groups, but also when comparisons were made between the 8-h time-point and the corresponding 4-h time-point, or with non-ventilated animals (blots not shown). The only ventilation group that did not exhibit lipocalin-2 expression changes between the 4-h and 8-h groups was the 10 cmH_2O PIP/2 cmH_2O PEEP group. It seems that all ventilation strategies were able to increase lipocalin-2 and Kim1 expression at the protein level in a time-dependent manner, but the strongest effect was observed for the 20 cmH_2O PIP/2 cmH_2O PEEP group.

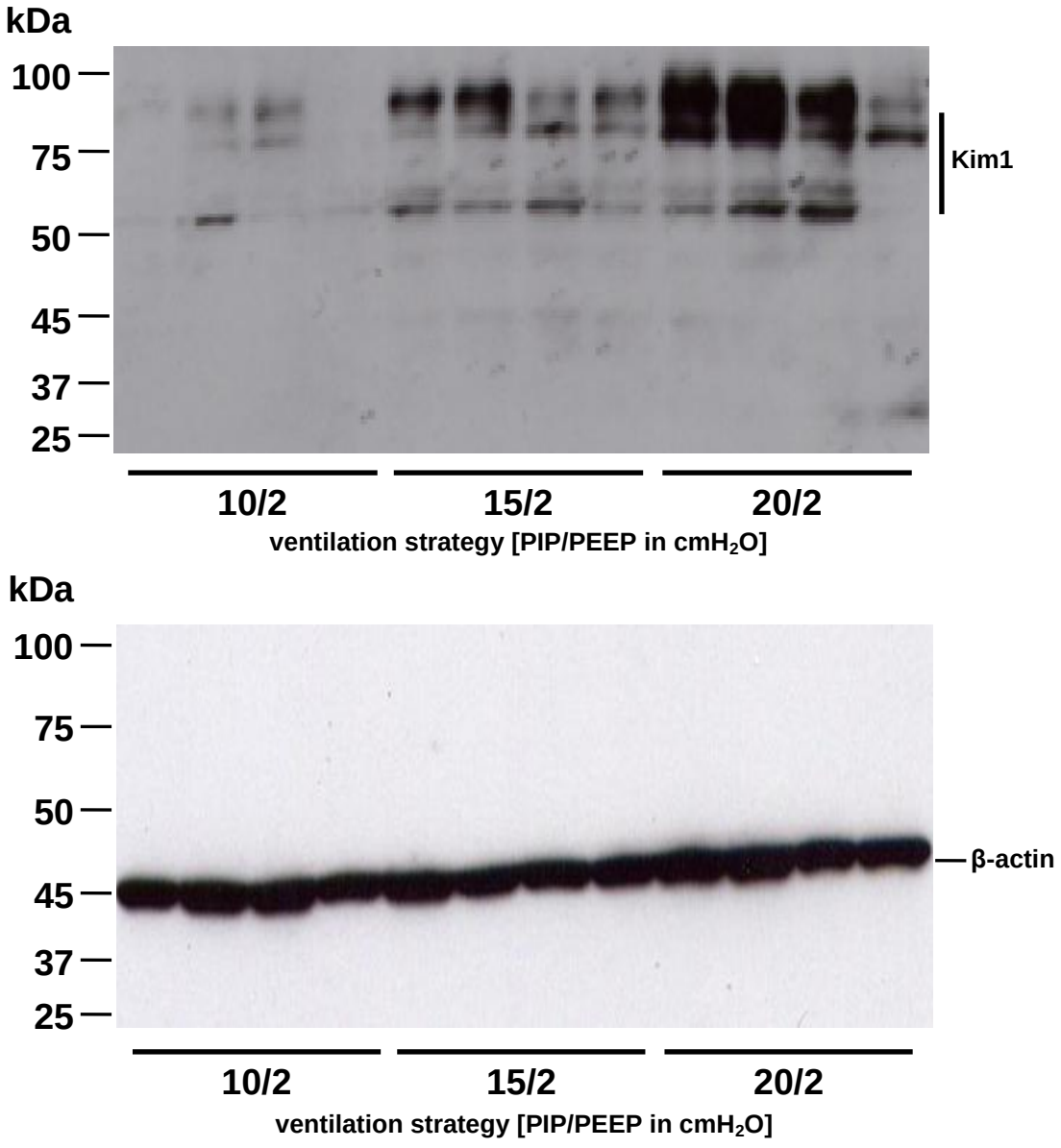

Figure 20. Kidney protein expression of Kim1. The protein expression of Kim1 (60-100 kDa) in kidney of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O) for 8 h. β-actin (45 kDa) served as a reference protein.

There is a discrepancy between the mRNA and the protein expression levels of lipocalin-2 obtained from kidney extracts and the urinary lipocalin-2 values. It seems that there are much larger changes at the mRNA and the protein level (especially in the 20 cmH_2O PIP/2 cmH_2O PEEP group) in tissue extracts, compared to the urinary lipocalin-2/creatinine levels. Lipocalin-2 must, therefore, be released from cells, pass through the urinary tract and hence, into urine. This process is delayed compared with changes in mRNA and protein level in the kidney, and is not, therefore, completely reflected by the values of lipocalin-2 observed in the urine.

3.11. Kidney morphology in mechanically ventilated rats

The H&E stain is a routinely used method for staining histological sections, utilising two compounds, haematoxylin and eosin to colour the tissue. Haematoxylin stains the nuclei of the cells blue, while eosin colours the cytoplasm red or pink.

In this study, H&E staining was used to examine kidney sections and to investigate whether any of the mechanical ventilation strategies were able to induce injury to the kidney. The light microscopy was used to visualise the kidney ultrastructure in the H&E sections. Mostly, this study concentrated on observations of acute tubular necrosis, recruitment of the immune cells to the kidney, and to some extent, vascular dysfunction (such as clot formation and endothelial disruption).

As expected, due to the short (8 h) experimental time-course, no dramatic changes to kidney ultrastructure were observed. Ventilated groups exhibited some differences compared to the non-ventilated animals (Figure 21). To some extent, some increases in immune cells recruitment, tubular necrosis, vascular occlusions, endothelial disruption and shading of the brush border could be detected.

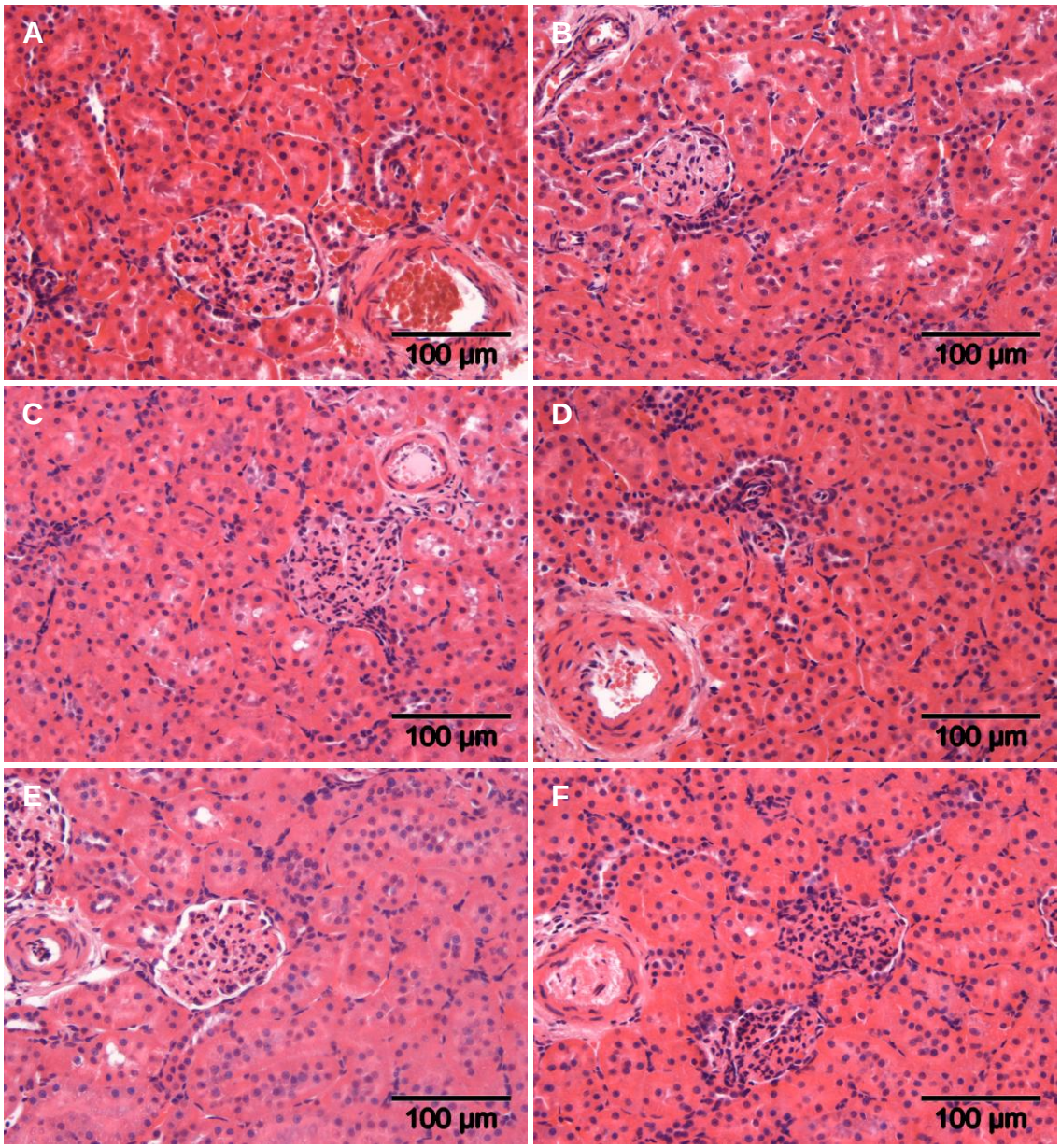

Figure 21. Kidney H&E staining. The H&E staining of the kidney of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O) for 4 h (B – 10/2, C – 15/2, E – 20/2 cmH_2O group) and 8 h (D – 15/2, F – 20/2 cmH_2O group). No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions.

3.12. Recruitment of immune cells to the kidneys of mechanically ventilated rats

Inflammatory processes play a central role in the development and progression of AKI. Macrophages, neutrophils and T-cells are known to be amongst the key players in the pathogenesis of AKI, and recruitment of inflammatory cells to the kidney can be one of the factors contributing to development of AKI. In these studies, the numbers of

immune cells were estimated in immunohistological sections of the kidneys from mechanically ventilated rats.

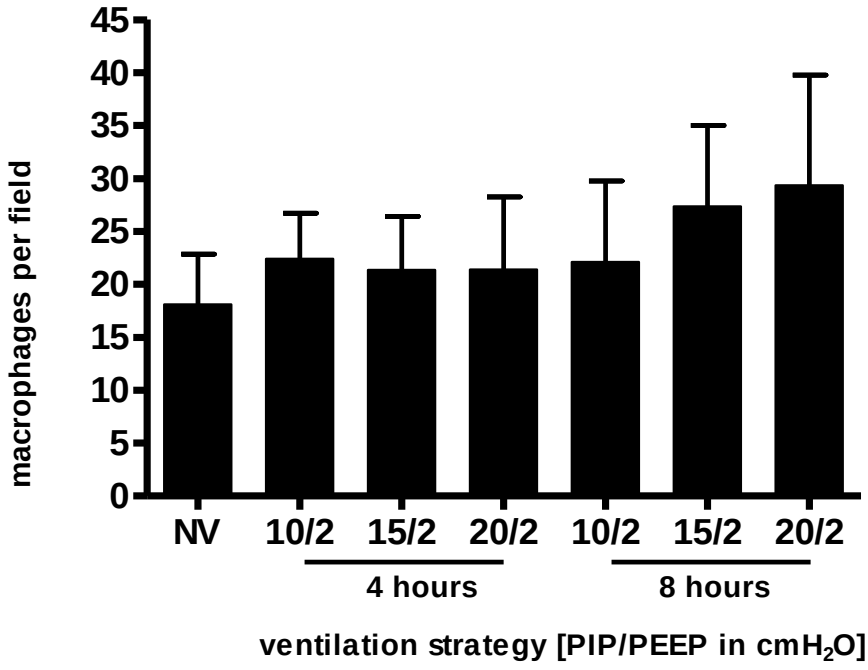

Figure 22. Quantification of macrophages in the kidneys. The number of macrophages in the kidneys of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, p<0.05; **, p<0.01; ***, p<0.001.

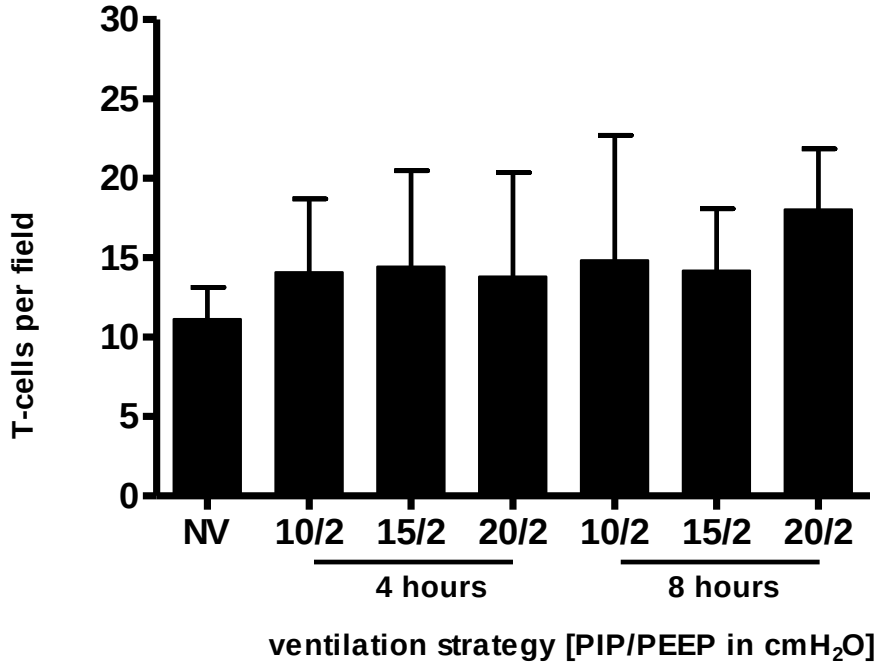

Figure 23. Quantification of T-cells in the kidneys. The number of T-cells in the kidneys of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, p<0.05; **, p<0.01; ***, p<0.001.

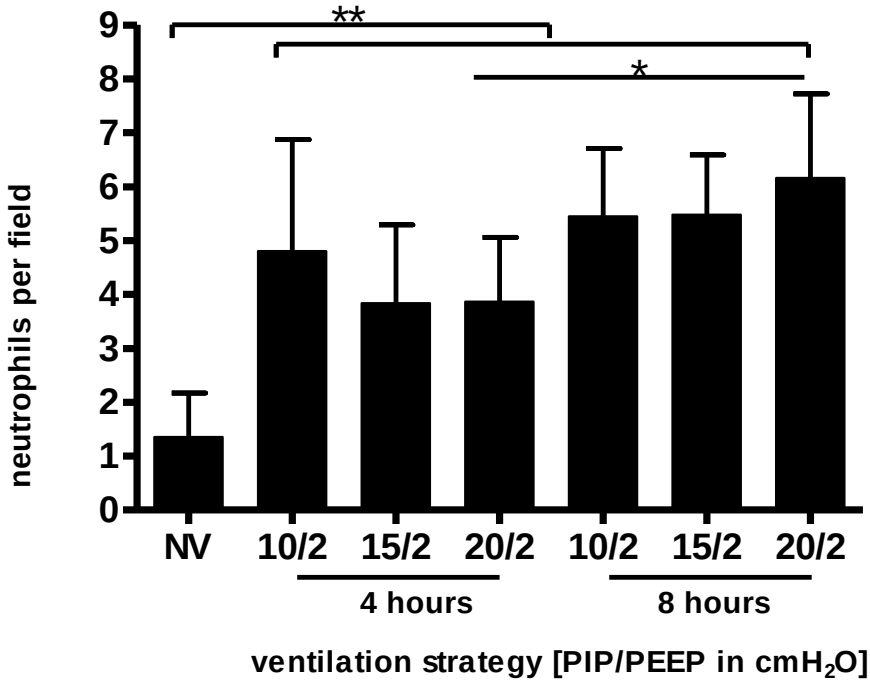

Figure 24. Quantification of neutrophils in the kidneys. The number of neutrophils in the kidneys of healthy rats ventilated with different peak inspiratory pressure (PIP) settings (10, 15, 20 cmH_2O) and constant positive end-expiratory pressure (PEEP) (2 cmH_2O). No manipulations were performed on non-ventilated (NV) animals indicating baseline conditions. Statistical comparisons were made by one-way ANOVA followed by the Newman–Keuls Multiple Comparison *post-hoc* test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

There were no differences observed in the recruitment of macrophages and T-cells of any mechanically ventilated groups (Figure 22, Figure 23, Figure 25, and Figure 26). These immune cells did not play a role in this animal model of mechanical ventilation. As illustrated in Figure 24 and Figure 27, this was not the case for neutrophils, where all ventilated groups exhibited statistically significant changes in the number of neutrophils (from three- to six-fold) *versus* non-ventilated group (Figure 24). The greatest increase in the recruitment of neutrophils could be observed for the 20 cmH_2O PIP/2 cmH_2O PEEP group. Additionally, there is a time-dependence only in the 20 cmH_2O PIP/2 cmH_2O PEEP group, where an elevation in the number of neutrophils was observed comparing 4-h and 8-h groups. It can be concluded that 20 cmH_2O PIP/2 cmH_2O PEEP group manifested the strongest inflammatory response in the kidney and is the most potent with respect to neutrophil recruitment and the development of AKI.

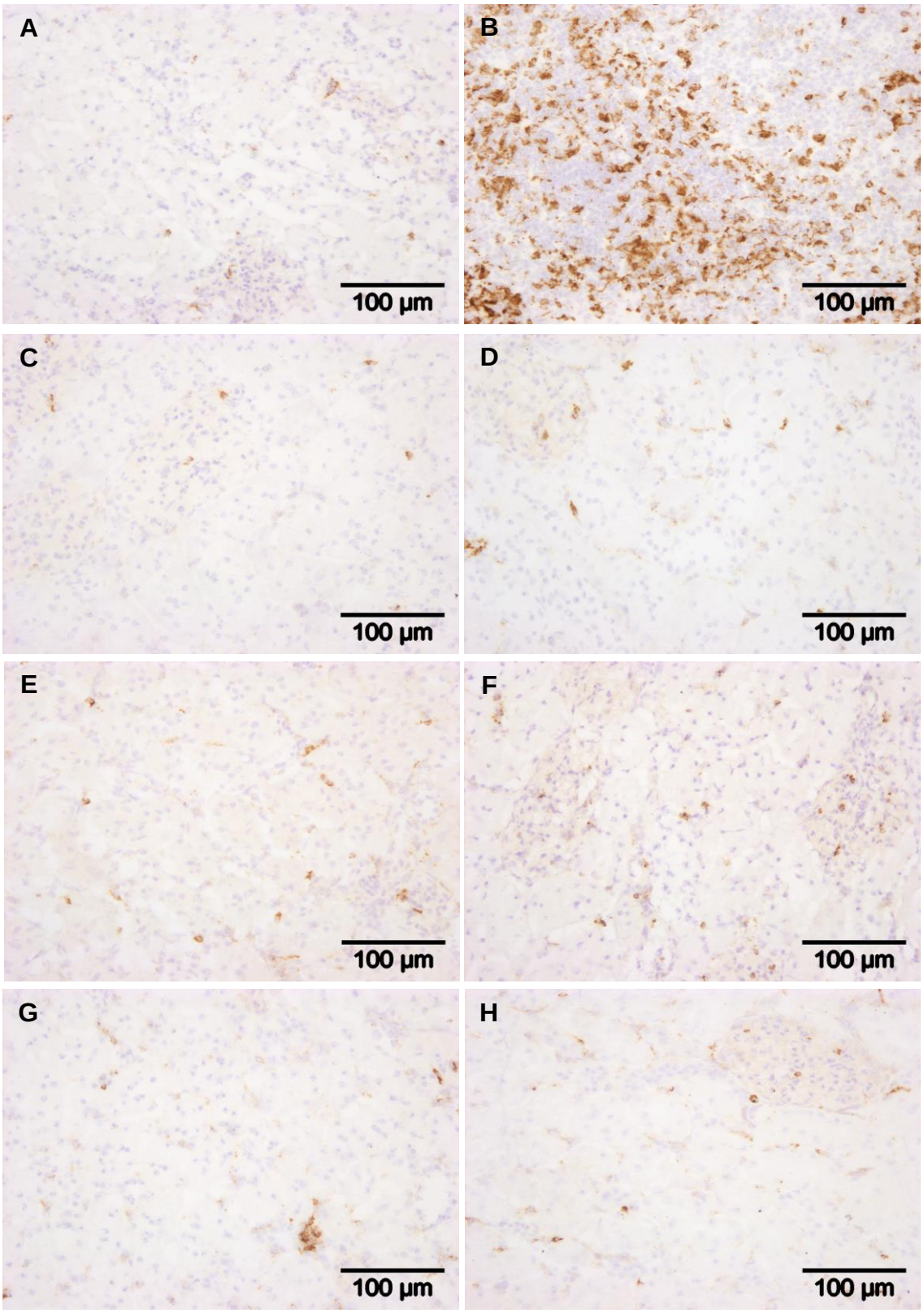

Figure 25. Detection of macrophages in the kidneys. Rats were ventilated at a constant positive end-expiratory pressure of 2 cmH_2O, with increasing peak inspiratory pressures of 10 cmH_2O (C, 4 h; D, 8 h), 15 cmH_2O (E, 4 h; F, 8 h) or 20 cmH_2O (G, 4 h; H, 8 h). Non-ventilated animals (A) indicated baseline conditions. Spleen staining (B) served as a positive control for macrophages.

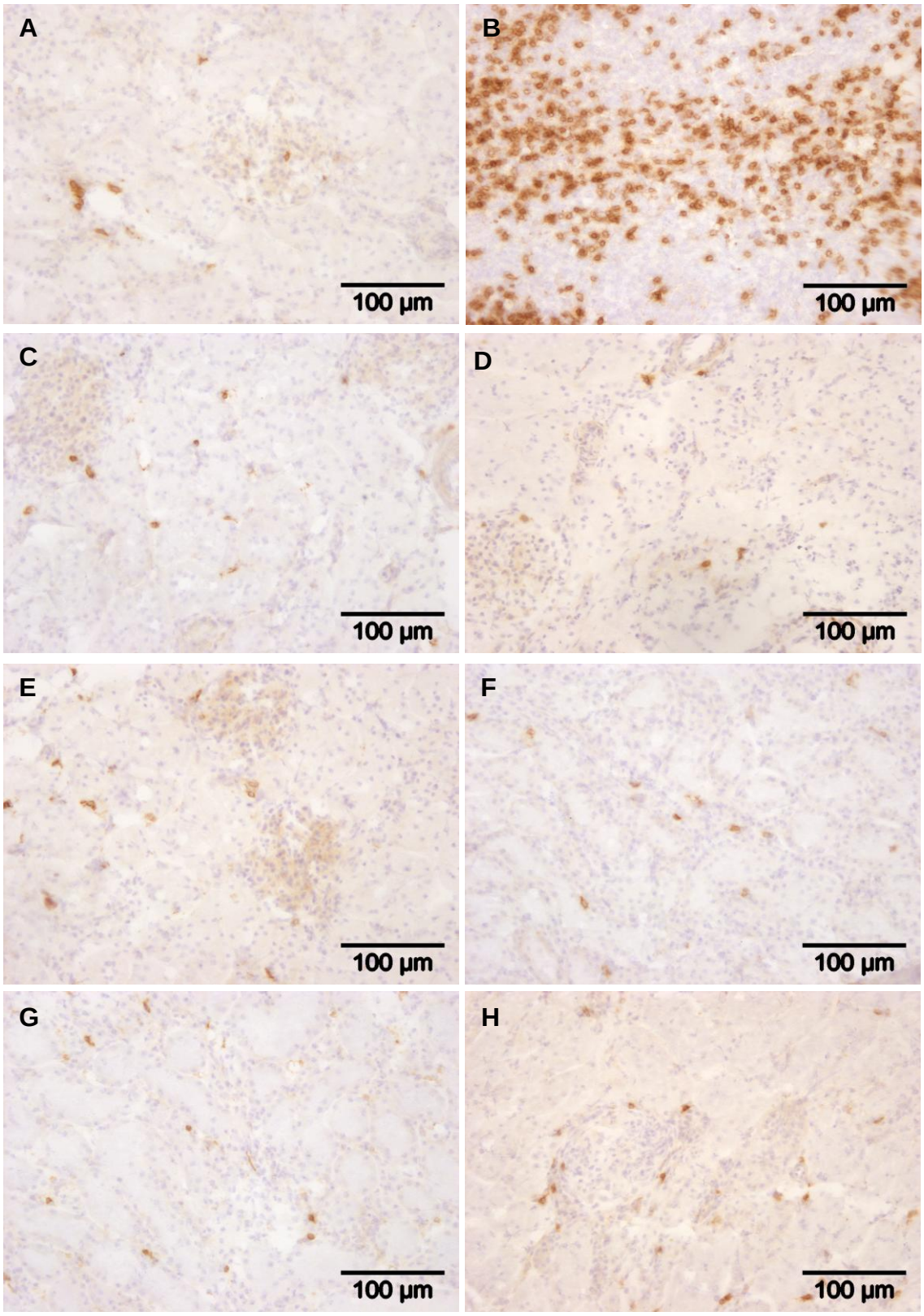

Figure 26. Detection of T-cells in the kidneys. Rats were ventilated at a constant positive end-expiratory pressure of 2 cmH_2O, with increasing peak inspiratory pressures of 10 cmH_2O (C, 4 h; D, 8 h), 15 cmH_2O (E, 4 h; F, 8 h) or 20 cmH_2O (G, 4 h; H, 8 h). Non-ventilated animals (A) indicated baseline conditions. Spleen staining (B) served as a positive control for T-cells.

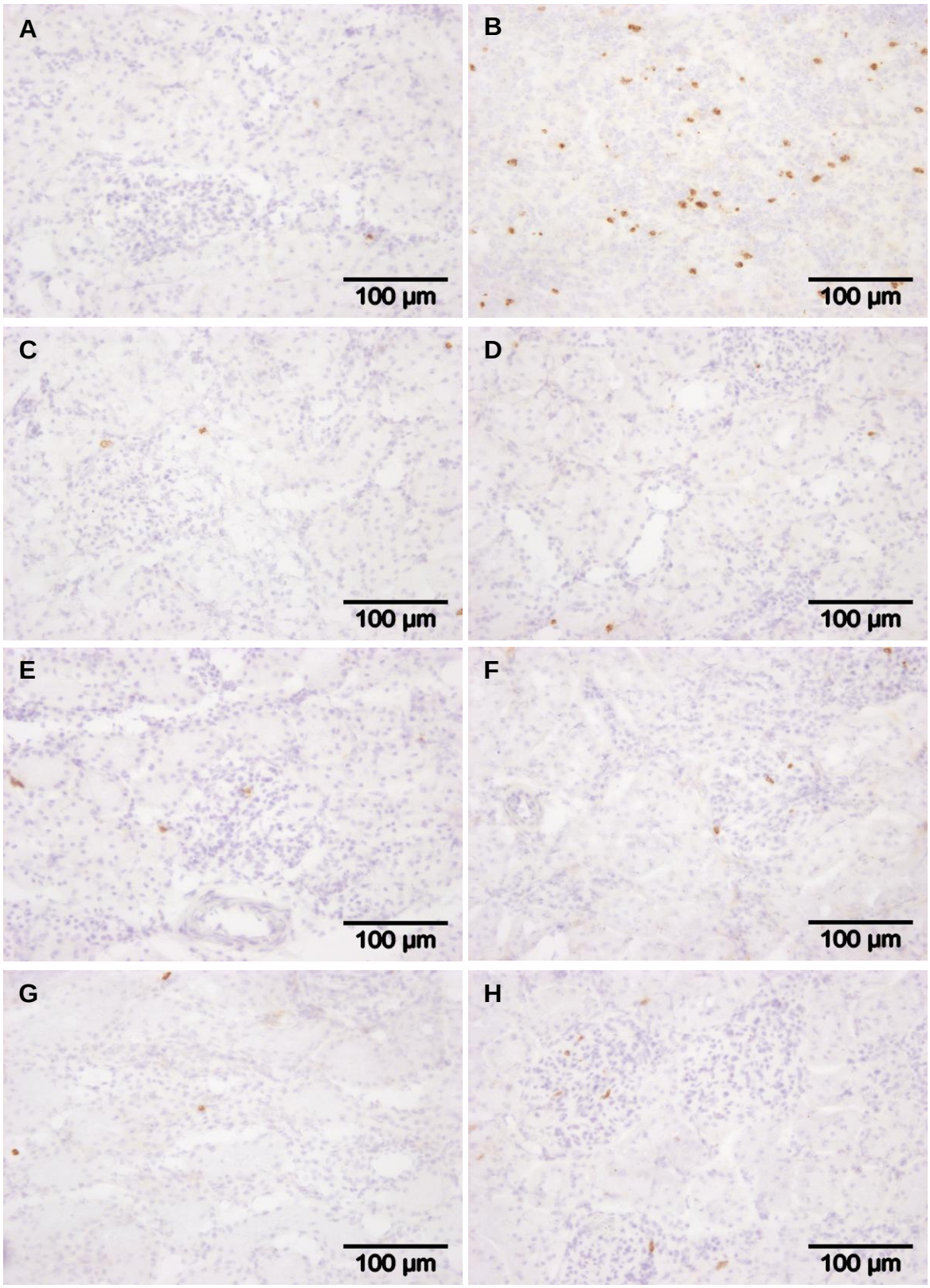

Figure 27. Detection of neutrophils in the kidneys. Rats were ventilated at a constant positive end-expiratory pressure of 2 cmH_2O, with increasing peak inspiratory pressures of 10 cmH_2O (C, 4 h; D, 8 h), 15 cmH_2O (E, 4 h; F, 8 h) or 20 cmH_2O (G, 4 h; H, 8 h). Non-ventilated animals (A) indicated baseline conditions. Spleen staining (B) served as a positive control for neutrophils.

4. Discussion

Most ICU patients must be mechanically ventilated. It has already been demonstrated that inappropriate mechanical ventilation is one of the factors that contribute to the development or worsening of ALI/ARDS. Although the influence of mechanical ventilation on lung function has been well described, a lot remains to be done to improve both the incidence and outcome of ALI/ARDS in ventilator-supported patients. It is known that improper mechanical ventilation can cause direct damage to the lungs. However, mechanical ventilation can exert its action not only on the lungs, but also through the lungs to distal organs, provoking the development of MODS. Multiple organ dysfunction syndrome is a clinical entity of much importance in an ICU setting, and is the subject of an increasing number of studies in the context of mechanical ventilation. Both clinical as well as experimental data suggest a direct interaction between the lungs and remote extra-pulmonary organs, and the impairment in lung-kidney communication is an emerging area of interest. Lung-kidney interactions appear to play a distinct role in ICU patients, leading to increased morbidity and mortality. A mortality rate of 30-40% is reached in ALI/ARDS patients admitted to the ICU. Noteworthy, a combination of ALI/ARDS and AKI leads to an increase in patient mortality, of up to 80% (Hassoun et al. 2007). In one study, it was demonstrated that low tidal volume ventilation (6 ml/kg) compared to high tidal volume ventilation (12 ml/kg) not only decreased the mortality from ALI/ARDS, but also decreased dysfunction in distal organs. In the first 28 days of treatment, patients treated with inappropriate (too high) tidal volume were associated with an increased number of days with renal failure compared with patients ventilated with a lower tidal volume. Additionally, studies performed on ventilated patients revealed that low tidal volume ventilation decreased the number of patients which developed kidney failure in the first 3-4 days of ventilator support (Hoag et al. 2008). Although some novel treatments for AKI are currently under consideration, such as poly(ADP-ribose) polymerase (PARP) inhibition, the use of lung protective mechanical ventilation strategy is regarded as the most important tool in clinical use that protect against the development of ALI/ARDS and its systemic insult with respect to MODS (Matejovic and Radermacher 2010, Singbartl 2011).

This study aimed to answer several questions regarding the development of AKI in response to mechanical ventilation. First, few mechanisms have been proposed to explain organ crosstalk, particularly between the lungs and the kidneys, in response to mechanical ventilation. Do these mechanisms include alterations to blood gases or

blood flow? Second, it is important to understand how mechanical ventilation affects the kidney with respect to the development of AKI. The possible response of the kidney to mechanical ventilation includes inflammation, with the release of cytokines and chemokines, recruitment of immune cells such as neutrophils, macrophages or T-cells, cell apoptosis and necrosis, and vascular dysfunction. This study also addressed the question of an appropriate biomarker of early AKI. To this end, lipocalin-2 and Kim1 were selected, because evidence presented in the literature supported the use of lipocalin-2 and Kim1 to monitor acute renal failure (Waring and Moonie 2011, Hall et al. 2011, Urbschat, Obermüller and Haferkamp 2011).

In this study, the time-dependence and the impact of mechanical ventilation on kidney structure and the kidney response to ventilation was investigated. Amongst the questions asked were: are 4 h or 8 h of mechanical ventilation sufficient to drive increased levels of markers of AKI? Are 4 h or 8 h of mechanical ventilation sufficient to provoke changes in kidney structure, recruitment of immune cells, and release of cytokines or chemokines? Is there a time-dependence with respect to the specific ventilation strategy applied?

Moreover, one of the aims of this study was to better understand the rat model of mechanical ventilation with respect to MODS, particularly AKI. Thus, additional questions asked were: are the changes associated with AKI that are observed in humans also reflected in small rodents? Can different mechanical ventilation strategies be correlated with different degrees of kidney damage? This study supports some known facts regarding renal inflammation in mechanically ventilated patients, but is one of the first studies to demonstrate that mechanical ventilation *per se* can impact leukocyte recruitment to the kidney of healthy animals.

The hypothesis of this study was that inappropriate mechanical ventilation is able to cause kidney injury in an animal model, and that there is a link between mechanical ventilation and the development of AKI. This hypothesis was tested in a rat model. Rats were mechanically ventilated for 4 h or 8 h, employing three different ventilation strategies: 10 cmH_2O PIP, 15 cmH_2O PIP and 20 cmH_2O PIP; with all groups having a constant PEEP of 2 cmH_2O. The assumption was that ventilation with 10 cmH_2O PIP/2 cmH_2O PEEP was the least damaging, and would not promote the same level of injury to the lung (and kidney) as would mechanical ventilation with 20 cmH_2O PIP/2 cmH_2O PEEP, which was expected to be the most injurious strategy, and was regarded as the strategy which would be suitable to define as an “inappropriate” or injurious ventilation. The “intermediate setting” of 15 cmH_2O PIP/2 cmH_2O PEEP was expected to yield an intermediate effect, permitting a dose-dependent analysis. The hypothesis also assumed that a short duration of 4 h

and 8 h of mechanical ventilation would exert a small effect on the kidney with respect to well-known markers of AKI (serum creatinine), but contrary to that, would have an effect on the expression of the novel potential and early markers of AKI (lipocalin-2, Kim1). The final assumption was that no changes would been seen in terms of the kidney structure (such as those changes revealed by H&E staining) after 4 h or 8 h of mechanical ventilation, but it would not be the case for molecular "initiator" responses, which should have already been altered.

New therapies that deal with the development of AKI and that reduce the mortality and morbidity from MODS are critically needed. Managing AKI by dialysis limits the symptoms rather than treats the disease. It is now postulated that the lack of sensitive and specific early biomarkers is one of the primary reasons why AKI is not detected early. Serum creatinine, a known marker of kidney injury, is late in response, and accumulates after tissue injury (Liu et al. 2007). A perfect biomarker should have perfect disease specificity (such as ischaemia-reperfusion *versus* nephrotoxic), prognostic capability (such as established *versus* acute AKI) and timing (such as early *versus* late in response). Many biomarkers have been proposed, however, the identification and validation of early biomarkers are problematic (Lisowska-Myjak 2010). First, the patient population in the ICU is very heterogeneous and hard to generalise. The question of prerenal, intrarenal or postrenal insult also has to be answered. Thus, the aetiology of AKI, and how and when it evolves, are unclear. Third, the incidence of sepsis in the ICU also limits the use of some biomarkers of AKI (Zappitelli et al. 2007). Most of the proposed biomarkers belong to processes or functions in inflammation, coagulation, tissue repair and others. Among many postulated new biomarkers, lipocalin-2 and Kim1 appear to be the most promising.

In this study, the observed changes in mRNA and protein expression levels, and in the tissue structure were small. This was anticipated, since AKI usually develops over several days, while the time-course employed in these studies was 8 h, which was a very short time-frame for biomarker generation in response to subtle stimuli (5 cmH_2O increments in PIP). Therefore, dramatic changes in lipocalin-2 and Kim1 expression were not expected. Nevertheless, some response in the kidney occurred, particularly at the molecular level. First, the levels of potential biomarkers of AKI were investigated. Indeed, a rapid increase in lipocalin-2 and Kim1 expression was observed when the most injurious mechanical ventilation of 20 cmH_2O PIP/2 cmH_2O was applied. The lipocalin-2 expression was more rapid than that of Kim1; however, both biomarkers revealed that the 20 cmH_2O PIP/2 cmH_2O ventilation strategy was the most damaging to the kidney. Importantly, a similar trend was observed at the protein expression level. A time-course investigation of these two potential biomarkers revealed a time-

dependence of the action of mechanical ventilation on the kidney, and supported the utilisation of lipocalin-2 and Kim1 as markers of kidney injury. The most injurious ventilation strategy of 20 cmH_2O PIP/2 cmH_2O stimulated the fastest response of the kidney to mechanical ventilation with respect to biomarkers of AKI, at the genomic as well as proteomic level. Statistical analyses revealed that the 10 cmH_2O PIP/2 cmH_2O group exhibited the smallest changes over the time-course of the experiment, while the 15 cmH_2O PIP/2 cmH_2O group exhibited transient changes, and the 20 cmH_2O PIP/2 cmH_2O group have the strongest response to mechanical ventilation, and the most injuriously affected kidneys.

In present study it is difficult to unambiguously conclude what mechanism of lung-kidney crosstalk during mechanical ventilation and the further development of AKI is most important. The mechanical ventilation strategy can influence blood gas parameters, which can then provoke changes in distant organs. In this study, we see that the “hyperoxic” conditions and hyper-inflation, exemplified by the most deleterious ventilation strategy of 20 cmH_2O PIP/2 cmH_2O, caused the most pronounced changes; increasing genomic and proteomic response of the kidney. However, ventilation with the 10 cmH_2O PIP/2 cmH_2O, which was regarded as under-ventilation, did not influence the kidney response as much as did the 15 cmH_2O PIP/2 cmH_2O and 20 cmH_2O PIP/2 cmH_2O strategies. Nevertheless, it has to be mentioned that these ventilation strategies created rather small “hyperoxic” or “hypoxic” conditions, in a very limited time. Thus, it is difficult to say how much these parameters affected the kidney. Nevertheless, mechanical ventilation with 20 cmH_2O PIP/2 cmH_2O provoked the largest changes. Blood gas measurements were supported by pH values, where tends toward respiratory alkalosis or acidosis were seen for 20 cmH_2O PIP/2 cmH_2O and 10 cmH_2O PIP/2 cmH_2O groups, respectively. However, the blood pressure (measured by MAP) remained within the physiological range. All three ventilation strategies did not appreciably impact the MAP, which remained over 80 mmHg, thus, the oxygen supply to the kidney should be adequate. Although the haemoglobin and lactate levels were altered by all three mechanical ventilation strategies, values remained within a range that would be considered physiological in a clinical analysis.

It is evident that mechanical ventilation leads to increased levels of total proteins in BALF. All three mechanical ventilation strategies drove up the level of proteins in BALF. Irrespective of the ventilator setting applied, every mechanical ventilation strategy provoked changes in lung function. Surprisingly, the most injurious ventilation, indicated by increased of total protein in the BALF, 20 cmH_2O PIP/ 2 cmH_2O, yielded “hyperoxaemic” blood oxygenation parameters. This suggests that

even during moderate damage to the lung, the blood oxygenation can be maintained or elevated, in the short term, so long as sufficient pressure, in this case PIP, is applied.

The H&E staining of kidney sections revealed that mechanical ventilation was able to alter kidney structure. This was evident by mild changes which suggested a low level of tubular necrosis, vascular occlusion and endothelial disruption.

It is known that mechanical ventilation can impact kidney function. One of the possible mechanisms is that inappropriate mechanical ventilation causes damage to lung structure with rupture of alveoli resulting in production and release of proinflammatory cytokines (biotrauma). Thus, the mechanical injury initiates the development of VILI, while inflammation is second insult. Therefore, a reduction of mechanical injury triggers less risk for VILI and MODS. However, mechanical ventilation triggers an inflammatory response in the lung, which can then initiate systemic and end-organ inflammation. It is known that cytokines such as IL-6, IL-8 and TNF-α are able to affect kidney function by promotion of neutrophil recruitment to the kidney, and stimulation of the release of leukocyte adhesion molecules (Broden 2009). Additionally, patients ventilated with lower tidal volumes exhibited a smaller increase in IL-6 and IL-8 levels in blood plasma. Thus, a protective ventilation strategy is thought to reduce the production of inflammatory mediators in the lung, and decrease the release of these mediators into the blood (Liu and Matthay 2008).

In present study it was demonstrated that mechanical ventilation was able to increase the number of neutrophils in the kidney. Neutrophils mediate tissue injury by releasing complex components of neutrophil granules which then can damage surrounding cells (Heinzelmann, Mercer-Jones and Passmore 1999). In this study it was demonstrated that AKI was associated with an increasing number of neutrophils in the kidney. However, the role played by neutrophils in the onset or development of AKI is controversial (He et al. 2008). One study has demonstrated that neutrophil depletion by anti-neutrophil serum in an ischaemic mouse model protected against the development of AKI (Kelly et al. 1996). Additionally, another study demonstrated that neutralisation of neutrophil-attracting chemokines such as Cxcl1 and Cxcl2, thereby inhibiting neutrophil recruitment to the kidney, attenuated renal ischaemia-reperfusion injury (Miura et al. 2001). Conversely, in an ischaemic AKI model, mice treated with the neutrophil-depleting rat IgG2b monoclonal antibody RB6-8C5 exhibited only small reduction in serum creatinine level and, despite a lack of neutrophils infiltrating the kidney, no decrease in the acute tubular necrosis score was observed (Melnikov et al. 2002).

The present study demonstrates that any ventilatory strategy used drove the recruitment of neutrophils to the kidney when compared to non-ventilated animals.

Additionally, only the most injurious ventilation strategy of 20 cmH_2O PIP/2 cmH_2O yielded a time-dependent effect with respect to the number of neutrophils recruited to the kidney. This suggests that these ventilator settings promote the recruitment of neutrophils compared to other ventilation strategies, and thus, was the strategy that most robustly impacted the kidney. In the present study, a pronounced increase in the number of kidney neutrophils was observed comparing the 4-h and 8-h time-points in the 20 cmH_2O PIP/2 cmH_2O group. Interestingly, the largest increase in the abundance of AKI biomarkers lipocalin-2 and Kim1 was also observed comparing these two groups. This study also demonstrates that mechanical ventilation of rats for 4 h or 8 h was able to induce changes in the expression of selected chemokines. It was also confirmed that in a time-dependent manner, the expression of Cxcl1, the chemoattractant for neutrophils, was also increased in abundance in the kidney after 4 h of mechanical ventilation.

T-cells serve as major modulators of the adaptive immune response. One of the most-studied models of kidney diseases is ischaemia-reperfusion injury. This model reflects the tissue injury found after transplantation, in chronic kidney disease, as well as in the acute phase of kidney failure. The T-cells can impair the function of the kidney epithelium, regulate the behaviour of other immune cells, as well as contribute to the release of inflammatory factors in the kidney (Rabb 2002).

Most of the studies which have investigated the role of T-cells in different experimental models of AKI have demonstrated the contribution of T-cells to the development of AKI (Rabb et al. 2000, Burne et al. 2001, Liu et al. 2006). Contrary to that, another study has demonstrated that ischemia-reperfusion kidney injury was not impacted when CD4 T-cells were depleted (Faubel et al. 2005). Thus, the role of T-cells in the onset of AKI remains to be elucidated.

Macrophages play a major role in inflammatory processes in all kidney diseases, once the immune response is activated (Duffield 2010). Many studies have demonstrated that macrophages are the major modulator of kidney injury in different experimental animal models such as ischemia-reperfusion injury, and reduction or elimination of macrophage activity contributes to protection against the development of AKI (Jo et al. 2006). However, in a cisplatin-induced injury model of AKI, the inhibition of macrophage recruitment to the kidney did not protect against the development of kidney injury (Lu et al. 2008). The latter observation possibly excludes the macrophage as a key mediator of AKI, and might suggest another critical component or immune cell type – such as the neutrophil – which could influence the development of AKI.

In this study, number of T-cells and macrophages were not altered comparing the experimental groups. It may be that the time-points investigated were too early to

provoke the recruitment of these immune cells to the kidney. Nevertheless, the observations suggest that among different types of immune cells, neutrophils are the earliest to respond to mechanical ventilation in the kidney, and recruitment occurs within 8 h of stimulation by mechanical ventilation.

Some challenges had to be addressed to study the influence of mechanical ventilation on the kidney in the rat model. The first problem arose with reagents that were essential to study urinary biomarkers of AKI. No specific ELISA kit for Kim1 is available, while the lipocalin-2 ELISA kit has recently been validated. Additionally, several technical issues had to be taken into account in the rodent model, including fluid support, and appropriate physiological monitoring. In this study, the mechanical ventilation model of ALI/ARDS was chosen to determine the kidney response to injury in the context of lung-kidney crosstalk. Many animal models of lung injury have already been established, however, none of these models completely recapitulates all of the features of clinical lung injury, which are characteristic for human ALI/ARDS. Importantly, to test the incidence of ALI/ARDS in animal models, the best parameters to measure are not physiological (lung compliance) but rather cellular responses such as neutrophil infiltration, histological assessment of lung structure, or indicators of alveolar-capillary barrier failure, such as BALF protein (Matute-Bello and Matthay 2011). A further concern is that rodents can only be mechanically ventilated for a few hours, while patients admitted to ICU have to be ventilated for days to weeks. It is difficult to estimate where features of human ALI/ARDS and the rodent models of ALI/ARDS meet, with respect to time-dependence. Another significant concern is related to the mechanical ventilation of healthy lungs and its clinical relevance, since in a clinical context, a health lung would not be mechanically ventilated for a substantial time period. Despite this concern, there is a need for a strong evidence of lung-kidney communication during mechanical ventilation, which can be more simply interpreted and understood without additional confounding factors, such as a background of inflammation or sepsis, which cause lung injury.

Several studies have been conducted to address concerns about the mechanical ventilation of healthy (non-injured) lungs. One study demonstrated that ALI/ARDS patients ventilated with high tidal volumes exhibited higher mortality, while patients chronically ventilated with large tidal volumes but without pre-existing ALI/ARDS, do not develop ALI/ARDS (Matute-Bello, Frevert and Martin 2008). Another study concentrated on impact of mechanical ventilation on the function of organs in healthy animals, where 15 piglets were randomised to one of three groups which included (i) spontaneously breathing, (ii) mechanically ventilated with tidal volume of 6 ml/kg for 12 h, and (iii) mechanically ventilated with tidal volume of 10 ml/kg group for

12 h. Mechanical ventilation was able to induce inflammatory processes in healthy animals. Additionally, higher tidal volumes exert their adverse effect not only on ventilatory parameters but also on distant organs (Kobr et al. 2010).

The present study explored the relation between ventilated lungs and the development of kidney injury, and focused on an examination of promising biomarkers of AKI, including lipocalin-2 and Kim1, and the elucidation of potential mechanisms behind the ventilator-induced kidney injury, particularly the inflammatory response. However, during the study, some new questions arose, which were not amongst the aims of the present study, but should be answered in subsequent studies. First, the present study did not focus on the localisation of lipocalin-2. Two independent, recent studies suggest the localisation of lipocalin-2 to the kidney epithelium at the onset of kidney injury (Cai et al. 2010, Paragas et al. 2011), however, these studies did not explore all possible AKI models, including ventilator-induced AKI. Thus, is it the kidney epithelium which over-expresses lipocalin-2 during mechanical ventilation, or another cell-type, perhaps neutrophils, which store lipocalin-2 in granules? The same question should be addressed regarding Kim1. The localisation of these two markers would provide knowledge about the cell-type which serves as the main source of biomarkers, and which responds to stimuli such as mechanical ventilation. Furthermore, the present study focused on the inflammatory response. It is also known that apoptotic events play a role in the development of AKI. It would be meaningful to combine these two elements, inflammation and apoptosis with respect to the influence of mechanical ventilation on the kidney. It would also be desirable to increase the ventilation time. At least 24 h of mechanical ventilation, with additional time-points in between, would probably result in more substantial damage to the kidney when inappropriate ventilation was applied. Additionally, the selected ventilation strategies (10/15/20 cmH_2O PIP/ 2 cmH_2O PEEP) are relatively close. Ventilation with larger PIP, such as 30 cmH_2O, would increase the level of kidney damage and the release of biomarkers. Although one might argue that healthy lung ventilation is not clinically relevant because healthy persons are never ventilated, the present study aimed to establish a starting point which explored whether mechanical ventilation - without additional injury - can provoke AKI. Additionally, further studies with mechanical ventilation and additional ALI/ARDS insults such as sepsis, acid aspiration or LPS challenge should be performed to compare the influence of mechanical ventilation alone or combined with additional systemic or pulmonary insults, on the development of AKI. Many studies have failed to answer the question of whether there is a soluble mediator which is produced by injured lungs that communicates with the kidneys, provoking damage. If such a mediator of injury could be found, significant progress in development novel anti-MODS

treatments or management strategies might ensue. The idea has been proposed that blocking such a mediator of injured lungs which communicates with the kidney might decrease morbidity and mortality from MODS. The questions of what the soluble mediators are and how much they affect the kidney in comparison to the effect of changes in physiological conditions such as blood gases or blood perfusion have to be answered. One approach to confirm the existence of such a mediator might be to harvest blood from animals ventilated with injurious ventilator strategies, and infusing this blood into healthy, unventilated animals, and assess whether AKI develops. It would also be worth investigating the genomic and proteomic response of mechanical ventilation to kidney throught utilisation of microarrays or proteomic approaches as well as microRNA studies for identification of early markers of AKI.

The present study was one of few which aimed to investigate how mechanical ventilation of healthy lungs affects the kidneys in a rodent model. It described the acute kidney response to mechanical ventilation, focusing on inflammatory processes. This study described in a time-dependent manner, which effects are exerted by mechanical ventilation on the kidney, highlighting new possible pathomechanisms at play which connect mechanical ventilation and the kidney response. Moreover, the present study associates neutrophil recruitment with the increased expression of candidate markers of AKI. The present study demonstrates that neutrophils are the first immune cells which respond in the kidney to mechanical ventilation. Additionally, this study supports the use of potential biomarkers, especially lipocalin-2, as a diagnostic tool in the ICU and describes the lipocalin-2 and Kim1 response in the kidney at the genomic and protein level.

In conclusion, proper ventilator settings and rapid diagnosis of kidney injury are key steps in the prevention and management of AKI in ventilated patients. Knowledge about which ventilation strategy is “bad”, and how this strategy impacts the kidney, are potential targets for further therapies, drug discovery, and management strategies. This study has laid some preliminary groundwork from which these ideas may be explored further, and which might contribute to a better understanding of the molecular mechanisms of ventilator-induced AKI.

5. Summary

Mechanical ventilation is the mainstay therapy for patients with acute lung injury (ALI) or the acute respiratory distress syndrome (ARDS), which exhibit a mortality of approximately 40%. Additionally, mechanical ventilation can also exert its action through the lungs to distant organs, provoking the development of multiple organ dysfunction syndrome (MODS). Thus, there is considerable interest in understanding ventilator-induced MODS with the aim to refine ventilation strategies, or augment supportive therapy, to attenuate organ failure in mechanically ventilated patients. This study focuses on acute kidney injury (AKI) because the kidney is one of the first organs affected by MODS. The goal of this study was to examine candidate biomarkers of AKI, and to contribute to our understanding of the pathophysiological mechanisms at play that result in MODS in response to mechanical ventilation.

Organ failure was induced in healthy rats by mechanical ventilation. Three different ventilation strategies were employed, using a pressure-controlled mode, with the positive end-expiratory pressure (PEEP) fixed at 2 cmH_2O, while the peak inspiratory pressure (PIP) was increased in 5 cmH_2O increments between 10 cmH_2O and 20 cmH_2O. All three ventilation strategies induced lung injury, as assessed by elevated protein levels in the bronchoalveolar lavage (BAL) fluid after 8 h mechanical ventilation. However, only the 20 cmH_2O PIP group displayed elevated BAL fluid protein levels after 4 h mechanical ventilation. No significant increase in serum creatinine levels at either time-point was noted in any of the experimental groups. Of the new, emerging biomarkers of AKI such as lipocalin-2 and Kim1, no significant increase in the mRNA expression levels of lipocalin-2 was detected in the kidney between 4 h and 8 h mechanical ventilation with the 10 cmH_2O PIP and 15 cmH_2O PIP strategies, however, pronounced changes were seen in the 20 cmH_2O PIP group. A comparable situation was observed for Kim1 expression. Comparing lipocalin-2 and Kim1, both exhibited a time-dependent increase in gene expression in the kidneys of mechanically-ventilated rats. Additionally lipocalin-2 appeared earlier, compared to Kim1, in the evolution of ventilator-induced AKI. There were no differences observed in the recruitment of macrophages and T-cells in any of the mechanically ventilated groups. However, an increase in the number of neutrophils was observed in the kidneys of rats ventilated with 20 cmH_2O PIP, where the greatest degree of lung damage, and the greatest increase in lipocalin-2 and Kim1 expression was also observed. These data suggest that mechanical ventilation can modulate neutrophil infiltration into the kidney.

6. Zusammenfassung

Die mechanische Beatmung ist die wichtigste Therapie für Patienten mit akuter Lungenschädigung (ALI) oder akutem Atemnot-Syndrom (ARDS), welche eine Mortalität von circa 40% aufweisen. Die mechanische Beatmung wirkt jedoch nicht ausschließlich auf die Lunge, sondern auch auf andere Organe. Dies kann zur Entwicklung eines Multiorganversagens (MODS) führen. Somit besteht erhebliches Interesse, die Vorgänge des beatmungsinduzierten MODS zu verstehen. Ziel ist es, die Beatmungsstrategien entsprechend zu verändern oder unterstützende Therapien zu verstärken, um ein MODS in mechanisch beatmeten Patienten zu verringern. Diese Studie beschäftigt sich mit akutem Nierenversagen (AKI), da die Niere zu den Organen zählt, die bei einem MODS als erstes betroffen sind. Das Ziel dieser Studie war es, bestimmte Biomarker der AKI zu untersuchen, um somit die pathophysiologischen Mechanismen einer beatmungsinduzierten MODS besser zu verstehen.

In gesunden Ratten wurde ein Organversagen durch mechanische Beatmung induziert. Es wurde jeweils ein druckkontrolliertes Verfahren, mit einem positiven endexspiratorischen Druck (PEEP) von 2 cmH_2O angewendet, während die maximale Kraft der Einatemmuskulatur (PIP) zwischen 10 cmH_2O, 15 cmH_2O und 20 cmH_2O variierte. Diese drei unterschiedlichen Beatmungsstrategien induzierten ein Lungenversagen, gemessen durch erhöhte Proteinwerte in der bronchoalveolären Lavage (BAL) nach 8 Stunden mechanischer Beatmung. Nach 4 Stunden mechanischer Beatmung zeigte nur die Gruppe mit 20 cmH_2O PIP erhöhte Proteinwerte in der BAL. Es wurde keine signifikante Erhöhung des Serumkreatinins zu beiden Zeitpunkten in einer der Versuchsgruppen festgestellt. Nach einer mechanischen Beatmung von 4 bis 8 Stunden bei einem PIP von 10 cmH_2O und 15 cmH_2O wurde keine signifikante Erhöhung der neuen Biomarker, Lipocalin-2 und Kim1, festgestellt. Deutliche Veränderungen konnten in der Gruppe mit 20 cmH_2O PIP ermittelt werden. Wenn man Lipocalin-2 und Kim1 vergleicht, zeigen beide eine zeitabhängige Erhöhung der Genexpression in den Nieren der mechanisch beatmeten Ratten. Des Weiteren erscheint Lipocalin-2 früher im Vergleich zu Kim1 bei der Entstehung einer beatmungsinduzierten AKI. Es wurden keine Unterschiede in der Anzahl der Makrophagen und T-Zellen in einer der mechanisch beatmeten Gruppen beobachtet. Jedoch konnte in den Rattenlungen, die mit einem PIP von 20 cmH_2O beatmet wurden eine Erhöhung in der Neutrophilenanzahl festgestellt werden. Diese Gruppe weist den höchste Grad der Lungenschädigung auf und somit auch den größten Anstieg der Lipocalin-2 und Kim1 Expression. Diese Daten weisen darauf hin,

dass durch mechanische Beatmung die Infiltration von Neutrophilen in die Niere reguliert werden kann.

7. List of abbreviations

AECC - The American-European Consensus Committee
ALI - Acute lung injury
AKI - Acute kidney injury
APS - Ammonium persulfate
ARDS - Acute respiratory distress syndrome
ARF - Acute renal failure
BAL - Bronchoalveolar lavage
BALF - Broncho-alveolar lavage fluid
BSA - Bovine serum albumin
BUN - Blood urea nitrogen
BW - Body weight
CBP - Cardiopulmonary bypass
cDNA - Complementary deoxyribonucleic acid
DAB - 3,3'-Diaminobenzidine
DEPC - Diethylpyrocarbonate
DNA - Deoxyribonucleic acid
dNTP - Deoxynucleotide triphosphate
DTT - Dithiothreitol
EDTA - Ethylendinitrilo-*N, N, N', N'*, -tetra-acetic-acid
EGTA - Ethylene glycol-bis (2-amino-ethylether)-*N,N,N',N'*-tetraacetic-acid
ELISA - Enzyme-linked immunosorbent assay
FABP - Fatty acid-binding protein
FiO2 - Fraction of inspired oxygen
H&E - Haematoxylin and eosin
HGF - Hepatocyte growth factor
HRP - Horse-radish peroxidase
ICAM - Inter-cellular adhesion molecule
I:E - Inspiratory:expiratory
IFN - Interferon
Ig - Immunoglobulin
i.m. - Intramuscular
i.p. - Intraperitoneal
IHC - Immunohistochemistry
ICU - Intensive care unit
IL - Interleukin

kDa - Kilodalton
Kim1 - Kidney injury molecule 1
LPS - Lipopolysaccharide
MAP - Mean arterial pressure
mmHg - Millimetre of mercury
MMP - Matrix metallopeptidase
mRNA - Messenger RNA
MODS - Multiple organ dysfunction syndrome
MV - Mechanical ventilation
NGAL - Neutrophil gelatinase-associated lipocalin
NV - Non-ventilated
O_2 - Oxygen gas
PA - Paraformaldehyde
PAI-1 - Plasminogen activator inhibitor 1
PaO_2 - Partial pressure of oxygen in arterial blood
PBGD - Porphobilinogen deaminase
PBS - Phosphate-buffered saline
PCR - Polymerase chain reaction
PEEP - Positive end-expiratory pressure
PIP - Peak inspiratory pressure
qPCR - Quantitative real-time polymerase chain reaction
RIFLE - Risk, injury, failure, loss, end-stage kidney disease
RNA - Ribonucleic acid
ROS - Reactive oxygen species
RR - Respiratory rate
RT - Room temperature
SD - Standard deviation
SDS - Sodium dodecyl sulfate
SDS-PAGE - SDS polyacrylamide gel electrophoresis
TAE - Tris-acetate-EDTA
TBS - Tris-buffered saline
TEMED - *N,N,N′,N′*-tetramethyl-ethane-1,2-diamine
TF - Tissue factor
TLR - Toll-like receptor
WB - Western blot
VILI - Ventilator-induced lung injury

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Table 4. List of primers used for qPCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Lipocalin-2	CTGGGCCTCAAGGATAACAA	CAGACAGGTGGGACCTGAAC
Kim1	TGGAGATTCCTGGATGGTTC	TAGTTGTGGGCCTTGTGGTT
PBGD	GGCGCAGCTACAGAGAAAGT	AGCCAGGATAATGGCACTGA
Ccl2	GCCAGATCTCTCTTCCTCCA	GAGTGGGGCATTAACTGCAT
Cxcl1	CCCCATGGTTCAGAAGATTG	AGCGTTCACCAGACAGACG

Table 5. List of primary and secondary antibodies.

Antibody	Host	Dilution		Company	Catalog number
		WB*	IHCH*		
Lipocalin-2	goat	1:250		R&D Systems	AF3508
Kim1	goat	1:250		R&D Systems	AF3689
β-actin	rabbit	1:2000		Cell Signaling	4967L
RP1 (neutrophils)	mouse		1:500	BD Pharmingen	550000
R73 (T-cells)	mouse		1:500	Serotec	MCA453G
ED1 (macrophages)	mouse		1:500	Serotec	MCA341R
PAP	mouse		1:100	Rockland	P200-0025
Anti-mouse secondary	rabbit		1:25	DakoCytomation	Z0259

* Abbreviations: western blot (WB), immunohistochemistry (IHCH).

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_____ _____

Ort, Datum Unterschrift

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