

Airway colonization, pathogenesis and prognosis in mechanically ventilated patients

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Abbreviations

VAP	ventilator associated pneumonia
ARDS	acute respiratory distress syndrome
SARS	severe acute respiratory syndrome
pdm (H1N1)	pandemic haemagglutinin1 neuraminidase 1 (influenza) virus
VAT	ventilator associated tracheobronchitis
BAL	bronchoalveolar lavage
PSB	protected specimen brush
RCT	randomized controlled trial
ATS	American Thoracic Society
IDSA	Infectious Disease Society of America
s-TREM-1	soluble triggering receptor expressed on myeloid cells
mRNA	messenger ribonucleic acid
CPIS	clinical pneumonia infection score
MRSA	methicillin resistant <i>Staphylococcus aureus</i>
CCCTG	Canadian Critical Care Trials Group
COPD	chronic obstructive pulmonary disease
MDR	multi drug resistant
ICU	intensive care unit
PCWP	pulmonary capillary wedge pressure
ALI	acute lung injury
NF- κ B	nuclear factor-kappa B
TNF- α	tumour necrosis factor-alfa
IL-1 β	interleukin-1 beta
Il-(n)	interleukin-(n)
Il-1ra	interleukin 1 receptor antagonist
TGF- β	transforming growth factor-beta
GM-CSF	granulocyte macrophage-colony stimulating factor
IP-10	interferon gamma induced protein 10
Th (n)	T-lymphocyte helper cells (n)
MCP-1	monocyte chemo attractant protein

MIP-1 α	macrophage inflammatory protein
PMN	polymorphonuclear leukocytes
H5N1	haemagglutinin 5 neuraminidase 1
HA	haemagglutinin
NS 1	non structural protein 1
NA	neuraminidase
PB 1	polymerase subunit 1
D222G/N	substitution of aspartic acid (D) in position 222 with glyc. (G) or aspar.(N)
NT-proANP	N terminal pro atrial natriuretic peptide
NT-proBNP	N terminal pro brain natriuretic peptide
SAPSII	simplified acute physiology score II .
SOFA	sepsis-related organ failure assessment
OR	odds ratio
PCR	polymerase chain reaction
FDR	false discovery rate
CD(n)	cluster of differentiation (n)
MMP-(n)	matrix metalloproteinase-(n)
S100-(n)	S 100 protein family
ANXA	annexin
KLRG/D1	killer cell lectin-like receptor subfamily G/D member 1
NFATC2	nuclear factor of activated T-cell
MHC II	major histocompatibility complex
IFN	interferon
IFI27	Interferon alpha-inducible protein 27
TCC	terminal complement complex
FGF	fibroblast growth factor
PDGF	platelet derived growth factor
VEGF	vascular endothelial growth factor
TIMP-(n)	tissue inhibitor of metalloproteinase

List of papers

This thesis is based on the following papers

Paper I

Berdal JE, Smith-Erichsen N, Bjornholt JV, Blomfeldt A, Bukholm G. Does *Pseudomonas aeruginosa* colonization influence morbidity and mortality in the intensive care unit patient? Experience from an outbreak caused by contaminated oral swabs. *Acta Anaesthesiol Scand* 2006 Oct;50(9):1095-102.

Paper II

Berdal JE, Bjornholt J, Blomfeldt A, Smith-Erichsen N, Bukholm G. Patterns and dynamics of airway colonisation in mechanically-ventilated patients. *Clinical Microbiology & Infection* 2007 May;13(5):476-80.

Paper III

Berdal JE, Stavem K, Omland T, Hall C, Smith-Erichsen N. Prognostic merit of N-terminal-proBNP and N-terminal-proANP in mechanically ventilated critically ill patients. *Acta Anaesthesiol Scand* 2008 Oct;52(9):1265-72.

Paper IV

Berdal JE, Mollnes TE, Waehre T, Olstad OK, Halvorsen B, Ueland T, et al. Excessive innate immune response and mutant D222G/N in severe A (H1N1) pandemic influenza. *J Infect* 2011 Oct;63(4):308-16.

1. Introduction

1.1 Invasive mechanical ventilation

Invasive mechanical ventilation is a lifesaving intervention for patients with acute, severe respiratory failure. Numerous medical conditions can lead to respiratory failure, but fall broadly into two categories: neuro-muscular disorders leading to hypoventilation, or processes within the lungs impairing gas exchange. The latter may originate in the lungs, such as severe pneumonia, autoimmune and degenerative lung diseases, or have extra pulmonary causes secondarily affecting the lungs, such as severe sepsis, acute pancreatitis and multiple trauma. Invasive ventilation is not without risks, and ventilator-associated pneumonia (VAP) is a major complication. VAP is, however, difficult to diagnose with certainty, and its attributable mortality is debated.

The lungs react in a stereotypical way to a range of insults. The term acute respiratory distress syndrome (ARDS) was coined acknowledging this, the definition being descriptive, involving a severe lowering of the ratio of inspired oxygen to oxygen in the blood, and bilateral chest X-ray infiltrates in the absence of cardiac disease explaining these abnormalities. Infections originating inside or outside the lungs, multiple trauma and abdominal catastrophes are common causes of ARDS. Bacterial infections are the most common, but viral infections also have the potential to cause ARDS. This was demonstrated during the Hantavirus Pulmonary Syndrome epidemic, the Severe Acute Respiratory Syndrome (SARS) epidemic of 2004, and, more recently, during the pdm(H1N1) influenza pandemic, where influenza remerged as an important cause of ARDS (1- 3) .

The main objectives of this thesis are to investigate airway microbiology, pulmonary infectious complications, and prognostic markers in patients on invasive mechanical ventilation, and to study host responses in ARDS caused by the new pdm (H1N1) influenza in particular.

1.2 Ventilator associated pneumonia

1.2.1 Pathogenesis.

Pneumonia occurs when a critical mass of sufficiently virulent bacteria reach the alveoli, overwhelms the local immune defence, multiply and invade neighbouring alveoli before immune cells recruited from the blood and lymphatic vessels can contain the process. VAP is the result of this sequence of events occurring in mechanically ventilated patients. Bacteria reaching the alveoli are a frequent event, and an autopsy study of patients dying of non-medical causes demonstrated oropharyngeal flora in the lungs of all the deceased (4). Micro aspiration of oropharyngeal fluids also occurs on a regular basis (5). In patients on invasive mechanical ventilation, a shift in the oropharyngeal flora occurs, introducing bacteria with possible higher virulence than the normal flora. The lungs are well equipped to deal with this continuous bacterial challenge, as they have a unique capacity to induce the innate immune response. Alveolar macrophages detect pathogens with the help of pattern-recognition receptors, phagocytise bacteria and, if overwhelmed, recruit the main effector cells of the innate immunity, the neutrophils (6). Antibacterial peptides are abundant throughout the lower airways, and play a role in killing bacteria before they reach the alveoli. The consequence of malfunction of this defence system is seen in cystic fibrosis. (7). The concept of a critical bacterial mass shifting the balance from a manageable challenge to pneumonia is recognized, and in various mouse models, inoculation of increasing concentrations of pneumococci in the alveoli, ultimately leads to pneumonia and death of the animals (8;9).

After endotracheal intubation, major defence mechanisms are broken, leading to an increase in the amount of bacteria reaching the alveoli. The intubation procedure itself frequently introduces a significant number of bacteria into the normally sterile distal airways. This is reflected in the clinical experience of a peak in the incidence of VAP of 3% per day in the first 4 days after intubation, thereafter falling to a daily incidence of 1% (10). The main bacterial reservoir feeding the distal airways, are the heavily contaminated oropharyngeal secretions accumulating above the endotracheal cuff, running into the lungs along the folds of the cuff surface and the tracheal wall of virtually all intubated patients (11). Detachment of bacterial colonies from endotracheal tube biofilm has been shown to occur regularly

during each inspiratory cycle, and these repeated bacterial exposures could overwhelm defences and lead to VAP (12). Contaminated condense water aggregating in the ventilator circuits can be inadvertently emptied or aerosolized into the endotracheal tube, but this mechanism, once thought to be a major source of airways contamination, probably plays a minor role (13;14). The mechanisms of clearing gross collections of fluids and mucus in the airways, such as cough reflex and mucociliar clearance, are heavily impaired by the endotracheal tube and sedation of the patient. Damage to the mucosa from the intubation procedure or from pressure and movement of the cuff during ventilation can denude the mucosa and expose bacterial binding sites (15). A state of relative immunosuppression following the inflammatory response of major surgery or sepsis is also contributing to increased vulnerability to infections, including VAP (16;17).

Ventilator-associated tracheobronchitis (VAT) has recently been proposed as an intermediate condition between airway colonization and VAP. The diagnostic criteria are fever, new or increased sputum production, positive cultures for tracheal aspirates, no other recognizable cause of fever, but with no radiographic infiltrate or evidence of pneumonia (18). In one study where these criteria were sharpened to include the requirement of tracheal bacterial counts of $> 10^6$ colony forming units (cfu)/ml, antibiotic treatment reduced both VAP incidence (13% vs 47%) and mortality (18 patients vs 47 patients) (19). The results from this study however, have not been reproduced. The jury is still out regarding both the existence of bacterial tracheobronchitis as a distinct entity, and the effect of antibiotic treatment, and it is possible that a significant number of VAT cases actually represent VAP with the “new or progressive infiltrate” not visible on poor-quality portable chest radiographs (20). A continuum from low, to high level colonization, to tracheobronchitis and VAP is however biologically plausible.

In summary, a shift in the oropharyngeal microbial flora, increased numbers of bacteria reaching the distal tracheobronchial tree, repeated episodes of bacterial inoculations, and reduced local and systemic defence mechanisms, all contribute to the frequent occurrence of pneumonia in the ventilated patient.

1.2.2 Diagnosis.

VAP is defined as pneumonia occurring more than 48 hours after intubation (21). It is suspected when an intubated patient develops new or progressive infiltrates on chest X-ray, fever, leucocytosis and purulent tracheal secretions (22). These clinical criteria however, lack both sensitivity and specificity, and over the past 20 years numerous rigorous and labour-intensive studies have been performed to improve diagnostic accuracy. Most efforts have been made investigating the value of including airway sampling and quantization of bacteria, to the clinical criteria of VAP (21;23). Defining the optimal sampling sites, endotracheal or distal airways, mode of sampling, whether bronchoscopic or blind, and defining bacterial count threshold values separating colonization from infection have been the principal focus of research (24;25). Bronchoscopic sampling techniques include bronchoalveolar lavage (BAL) or protected specimen brush (PSB), with quantitative culture limits for differentiating bacterial infection from colonization of $\geq 10^4$ cfu/ml for BAL, and $\geq 10^3$ cfu/ml for PSB. There is much controversy about the usefulness of this diagnostic approach. In 2004 the current state of affairs was summed up in an editorial : “Over 300 studies have been published in peer-review journals in the past 8 years dealing with management of ventilator-associated pneumonia (VAP). However, no consensus exists to date on the best way of identifying patients with true lung infection.” (26). Seven years later, this statement is still valid. The lack of a gold standard for the diagnosis of VAP is a major obstacle to the design of studies aiming at improving diagnosis, and it is unlikely to be solved in the foreseeable future. Even the presumed histopathological gold standard of VAP has inherent limitations, such as sampling errors, inability to assert the age of the pneumonic lesions, and the bias of only being able to include deceased patients, or the rare patient upon whom an open lung biopsy has been performed. Enthusiasm for invasive sampling for diagnostic purposes has also been hampered by lack of evidence of improved outcome (27). A large randomized controlled trial (RCT) comparing invasive and non-invasive diagnosis for VAP, demonstrated reduced use of broad spectrum antibiotics, reduced 14 days mortality, and reduced organ failure at day 7 in the invasive diagnosis arm, but no difference in 28 days mortality (28). Furthermore, the other favourable results of the study were questioned as only one patient in the invasive diagnosis group received initial inadequate empiric antibiotic therapy versus 24 in the non-invasive group, thus possibly overestimating the effect of the intervention and underestimating the effect of inadequate therapy (29). With accumulating evidence of the role of early adequate antibiotic therapy as

the main prognostic factor in VAP (27;30-32), there is an increased recognition of the limitations of distal airway sampling and quantitative cultures for diagnostic purposes, as the result will arrive too late to influence treatment decision and initial antibiotic choice (33). An exception is information gained by direct microscopy as this could be used to rule out VAP if no bacteria are seen. In the influential American Thoracic Society / Infectious Disease Society of America (ATS/IDSA) guidelines on VAP, this point is taken into the decision tree on when to start or withhold treatment (34). However, other direct microscopy criteria have also been proposed, such as percentage of neutrophils to total leukocytes, or, the number of BAL cells containing intracellular bacteria (35). Neither has been validated, and for the latter, widely differing thresholds of 1%, 5%, or 7%, respectively, have been suggested (35;36). In clinical practice, concerns about sampling or processing errors, as well as the clinical judgment of the seriousness of the patient's condition are likely to overrule treatment decisions based on microscopic findings. Distal airway sampling before commencing antibiotic treatment is still important in order to adjust antibiotic treatment when culture results become available as well as for re-evaluating the VAP diagnosis when uncertain and alternative diagnosis may have emerged since initiation of therapy. The microbiological epidemiology may vary considerably at country- and hospital level, sampling for surveillance purposes is, therefore essential for choosing adequate local empirical antibiotics regimens (37). Blood cultures and cultures of pleural fluids are important specimens for diagnosis, but are rarely positive in VAP, and as with airways samples, results come too late to influence initial therapy (38). Given the rarity of positive blood cultures in VAP, especially regarding gram-negative bacteria, this finding should prompt investigations of alternative sites before accepting the lungs as the source (39). The limitations of the bacteriological criteria for diagnosing VAP have led to search for alternative strategies, notably exploring host immune responses. Soluble triggering receptors expressed on myeloid cells (s-TREM-1), which are up-regulated on the surface of inflammatory cells in the presence of bacterial infections have been investigated as a potential marker of VAP. In 2004 a study was published where s-TREM-1 in BAL fluid performed favourably compared to combined clinical and microbiological criteria for diagnosing VAP (40). Several similar studies have been published since, some using serial measurements of s-TREM-1, or another widely investigated biomarker, pro-calcitonin (22;41). They all share the inherent limitation of the field, the lack of a gold standard for VAP diagnosis, thus, a biomarker-based approach merely speeds up diagnosis by bypassing

time-consuming bacterial cultures, not improving diagnostic accuracy as such. Schuetz et al. summarized in a recent review the way forward in biomarker research in infectious diseases: “only randomized controlled trials, in which antimicrobial therapy is guided by specific cut off ranges of the biomarkers and in which the primary measure of efficacy is medical outcome, have the potential to evaluate the ultimate clinical usefulness of a diagnostic biomarker” (42). This is also valid for biomarker studies using mRNA gene expression signatures of BAL or peripheral blood cells, to differentiate infections from other causes of respiratory failure. Although in its infancy, identification of a set of genes expressed only or predominantly in infections is promising (43). The merit must be evaluated as for other biomarkers, but the sheer number of simultaneously investigated genes with micro array technology promises better differentiation between various clinical similar, but pathological different conditions, as opposed to current investigation of single gene products. This approach has already been successful in oncology, where clinical and histological similar cancers have been subdivided into a number of diseases with different prognosis and treatments (44).

A formalized clinical evaluation in the form of a Clinical Pneumonia Infection Score (CPIS) has also been proposed to diagnose VAP (23). The scoring system assigns points based on 6 clinical assessments, each worth 0 –2 points, including: fever, leukocyte count, quantity and purulence of tracheal secretions, oxygenation, type of radiographic abnormality, and results of sputum culture and Gram stain. When applied prospectively, sputum culture and Gram stain are unavailable and a modified scoring system, omitting culture results has been proposed by Singh et al. (45). In their study, aimed at reducing antibiotic overuse, antibiotics were safely discontinued if the CPIS score remained < 6 on day 3. For the day 3 CPIS score calculation, results of culture and Gram stain were included. The aggregate score of CPIS is, however, very similar to a clinician using all available data to decide how strongly the diagnosis of pneumonia is suspected. The use of clinical criteria to initiate, and microbiological data to modify or terminate antibiotic therapy is now accepted as a standard of care (46).

1.2.3 Treatment.

The most important factors for outcome of VAP are early and adequate antibiotic therapy. Starting with a wrong antibiotic cannot be reversed by later antibiotic adjustments according

to sensitivity testing, although this was recently contested in patients with less severe disease (27;30;32;47;48). The mainstay of therapy for VAP is therefore awareness, rapid diagnosis, and prompt treatment initiation, choosing an empiric antibiotic therapy that is appropriate to the local microbiological epidemiology. No single antibiotic or combination of antibiotics can be recommended over others, but activity against the core common bacteria: streptococci, *S.aureus* and enterobacteriaceae, are necessary. The need to add coverage for methicillin resistant *S. aureus* (MRSA), *P. aeruginosa* and other non-fermentors or multi-drug resistant enterobacteriaceae, will depend on how likely these microbes are encountered locally. It is generally not considered necessary to give coverage for anaerobes or the frequently isolated *Candida* species (49). De-escalation to less broad coverage is recommended when culture results become available (50). One large RCT found 8 days therapy to be equally effective to 15 days (51), and several studies have proposed criteria for early antibiotic discontinuation (52-54). ATS guidelines (1995) recommending 7-10 days for uncomplicated and 14-21 days for more serious infections were not based on prospective studies (55). Copious airway secretions are removed with tracheal suction when necessary, but should not be routinely scheduled. Chest physiotherapy, secretion removal with bronchoscopy, and coughing machines are widely used, but their impact is debated (56).

1.2.4 Prognosis

Crude mortality is high in ventilated patients, but assessment of mortality due to VAP, is complicated by the difficulty of precisely establishing the VAP diagnosis, and separate the VAP mortality from the mortality due to underlying disease. This problem increases with severity of underlying illness as the sickest patients will have the highest mortality, be the most vulnerable for acquiring VAP, and tolerate it the least. Thus, depending on patient population and VAP definitions, different figures of VAP-attributable mortality prevail. Craven et al reported 55% mortality in 49 patients treated with mechanical ventilation for nosocomial pneumonia, Kollef reported 37%, and Fagon et al. 71 % (57-59), the latter using PSB for diagnosis. Using a rigorous method matching ICU patients with and without pneumonia, VAP- attributable mortality was found to be 27 % (60). In the same study, pneumonia due to *Pseudomonas* and *Acinetobacter*, showed even higher attributable mortality rate of 42 %. Higher mortality due to these pathogens has also been demonstrated in other studies (61). Patient category also matters, no increased mortality of VAP was

found in trauma patients (62), and no difference in mortality was found for ARDS patients with or without concomitant VAP (63). Thus the impact of VAP is difficult to detect in conditions with low mortality, as in trauma or with high mortality, as in ARDS (38). An increased mortality in medical versus surgical patients was found in the Canadian Critical Care Trials Group (CCCTG), with a relative risk increase of 65% versus 27% (27). In this trial, the largest case-control study of mortality attributable to VAP, an absolute risk increase of 5, 8%, or a 33% relative risk increase of death was found, but the results did not reach statistical significance. The 27% VAP-attributable mortality found by Fagon and coworkers (60) versus zero VAP-attributable mortality in the study of Papazian et al. may also be due to the difference in the proportion of medical patients, 44% and 26% respectively (64).

In conclusion, risk of death and risk of getting VAP covariate, posing inherent methodological problems delineating the mortality impact of VAP. Definite answers to VAP-attributable mortality would involve ethically unacceptable controlled studies withholding antibiotic treatment for VAP. The 33% relative risk estimates of the CCCTG probably reflect the best possible estimates with current diagnostic tools.

1.3 Airway microbiology in mechanical ventilated patients

Johnson et al. described, in 1969, the changing pharyngeal bacterial flora in hospitalized patients (65). They observed a marked increase in the prevalence of gram negative bacteria, not correlated to antibiotic exposure or length of hospitalization, but best correlated with the severity of underlying disease. Their findings were reproduced, and the causes for this transition were investigated, in several following papers (66-69). Niederman proposed three factors influencing microbial binding: epithelial cell variables, micro environmental variables, and bacterial variables (66;70).

Fibronectin, an ubiquitous mammalian glycoprotein with diverse biological functions related to cell adhesion, has been implicated in confining epithelial attachment for the streptococci of normal oropharyngeal flora (71). Fibronectin is secreted with saliva and crevicular fluid (ultra filtrate of serum secreted through oropharyngeal mucosa), and then coat the oropharyngeal mucosa (72). Loss of fibronectin has been shown to be important in enabling gram negative bacteria to adhere to, and colonize the oropharyngeal mucosa (71;73-75).

There are two possible explanations for the decreased epithelial fibronectin: Increased release of sputum leukocyte-derived proteases, and to a lesser extent, gram-negative bacteria-derived proteases, lead to increased cleaving of fibronectin from epithelial cell surfaces. In sick patients there is a reduced secretion of fibronectin from saliva and crevicular fluid (75). The importance of fibronectin on buccal cells has, however, been contested, and one study reported absence of fibronectin on normal buccal mucosa (76). Weinmeister et al. argued that a reduction of cell surface carbohydrates, notably galactose and sialic acid, is responsible for the increased binding of gram-negative bacteria. Though the responsible gram-negative bacteria binding cell surface structures are disputed and probably involves multiple bacterial adhesins and mucosal surface receptors, the concept of a cell surface alteration caused by increased activity of respiratory tract proteases in the sick and more so in the critically ill, is similar (77).

The micro-environmental changes caused by intubation are numerous. The foreign body surface of the endotracheal tube allows the formation of bacterial biofilms in which bacteria are protected from the immune system and largely unresponsive to antibiotics (12;78). The endotracheal cuff pools heavily contaminated fluid above the cuff, and causes damage to the mucosa, exposing binding sites for bacteria. Disruption of mucociliar function promotes prolonged mucosal contact with bacteria needed for colonization to occur. The endotracheal tube thus leads to an increase in both numbers and occasions for bacteria to seed the lungs. Lower airway colonization is also seen in non-intubated patients with micro-environmental changes and impaired mucocilar function, such as the *H.influenzae* colonization seen in chronic obstructive pulmonary disease (COPD), or the *P. aeruginosa* colonization seen in cystic fibrosis. *P. aeruginosa*, has been shown to adhere poorly to intact ciliated epithelium, but adheres to damaged epithelium and basement membrane (79), possibly accounting for its frequency in VAP. A dynamic interaction between airway cells and bacteria has also been shown in a study with *S.aureus*; one hour after exposure, adherence to respiratory epithelium was low and inoculum independent, without damaging epithelial cells. However, at 24 hours more bacteria adhered, internalized bacteria were observed and a bacterial concentration-dependent cell necrosis occurred, suggesting a capacity for early defence but an increasing imbalance in host-pathogen interaction after prolonged bacterial exposure, such as seen in ventilated patients (80).

The bacteria recovered from endotracheal aspirates and BAL fluids are similar across several studies (28;62;63;81) see table 1. Local variations are considerable and surveillance cultures are mandatory for choosing appropriate local empiric regimens (37).

TABLE1

ETIOLOGY OF VENTILATOR-ASSOCIATED PNEUMONIA
AS DOCUMENTED BY BRONCHOSCOPIC TECHNIQUES IN 24
STUDIES FOR A TOTAL OF 1,689 EPISODES AND 2,490 PATHOGENS

Pathogen	Frequency (%)
<i>Pseudomonas aeruginosa</i>	24.4
<i>Acinetobacter</i> spp.	7.9
<i>Stenotrophomonas maltophilia</i>	1.7
Enterobacteriaceae*	14.1
<i>Haemophilus</i> spp.	9.8
<i>Staphylococcus aureus</i> [†]	20.4
<i>Streptococcus</i> spp.	8.0
<i>Streptococcus pneumoniae</i>	4.1
Coagulase-negative staphylococci	1.4
<i>Neisseria</i> spp.	2.6
Anaerobes	0.9
Fungi	0.9
Others (< 1% each) [‡]	3.8

* Distribution when specified: *Klebsiella* spp., 15.6%; *Escherichia coli*, 24.1%; *Proteus* spp., 22.3%; *Enterobacter* spp., 18.8%; *Serratia* spp., 12.1%; *Citrobacter* spp., 5.0%; *Hafnia alvei*, 2.1%.

[†] Distribution when specified: methicillin-resistant *S. aureus*, 55.7%; methicillin-sensitive *S. aureus*, 44.3%.

[‡] Including *Corynebacterium* spp., *Moraxella* spp., and *Enterococcus* spp.

A distinction between early and late VAP, commonly defined as VAP occurring before or after 4 days of intubation, has in several studies been proposed to distinguish between likely non-multidrug resistant organisms in the former, and possible multidrug resistant (MDR) bacteria (MRSA, Extended Spectrum Beta Lactamase- and Carbapenemase producing gram-negative bacteria, Vancomycin Resistant Enterococci) in the latter (82). This distinction is based on the assumption that the longer the intensive care unit (ICU) stay, the greater the chance of acquiring ICU domestic MDR bacteria. The concept of early and late VAP has, however, been questioned, and no clear distinction between microbial findings were found in a recent large study (83). It is conceivable that knowledge of local microbiology offers a more powerful tool to decide whether empirical coverage for MDR organisms is required in individual ICUs (83). The source of the flora colonizing the airways in ventilated patients has been extensively investigated. The upper gastrointestinal tract, the sinuses, the circuits and tubes of the ventilator system, attending nursing personnel hands, have all been implicated (84). A consensus has evolved, and there is acceptance that the patients own airway and gastrointestinal flora is the reservoir for seeding of the distal airways (85). The composition of this reservoir changes according to the pathogens prevalent in the ICU and the effectiveness of hygiene measures in preventing acquisition of “in-house” microbes (86). The propensity to establish a gram-negative flora, and even more opportunistic bacteria, in the airways, is correlated to seriousness of underlying disease (87). Anaerobes, representing the majority of bacteria of both the upper and lower gastrointestinal tract, have long been covered in empiric regimes of VAP and in aspiration pneumonias. Several papers have questioned the necessity of anaerobe coverage and it is biologically plausible, that the aerobic environment created by the high oxygen concentrations delivered with mechanical ventilation, cannot promote anaerobe proliferation and infection (49). The need to treat *Candida* species, often recovered from airway samples in ventilated patients, has also been debated. Several well-designed studies have shown that the invasive potential of *Candida* in the airways is limited (88;89), and when *Candida* pneumonia occurs, it is in the context of haematogenous seeding after profound and prolonged neutropenia.

1.4 The pathophysiology and inflammatory response of Acute Respiratory Distress Syndrome (ARDS).

The adult respiratory distress syndrome (ARDS) was described in 1967 in a dozen patients with refractory hypoxemia and diffuse lung infiltrates, the authors at the time recognizing that different insults could lead to the syndrome (90). In contrast to the neo-natal respiratory distress syndrome, lack of surfactant production from immature lungs is not the cause, and surfactant supplement not beneficiary. *Acute* respiratory distress syndrome is now the preferred term, recognizing that the same type of increased permeability oedema also occurs in children (91;92). In 1994, an expert group proposed an operational definition of ARDS, consisting of bi-lateral diffuse alveolar chest x-ray infiltrates, $\text{PaO}_2/\text{FiO}_2$ ratio < 27 kPa, in the absence of elevated left atrial pressure measured as pulmonary capillary wedge pressure with a pulmonary catheter (PCWP < 18 mm Hg) when measured, or without clinical evidence of left atrial failure (93). Acute Lung Injury (ALI) was defined similarly, but on the less severe end of respiratory failure the $\text{PaO}_2/\text{FiO}_2$ ratio was set at 40 kPa. They further stated that ARDS is characterized by a constellation of clinical, radiological and physiological abnormalities that cannot be explained by, but may co-exist with, left atrial or pulmonary capillary hypertension. The definition is a tool for clinicians and scientists conducting research, but is not helpful for discriminating causes, or prognosis of ARDS. An acute and excessive inflammatory response driving the pathological changes to the lung parenchyma is at the core of ARDS (94). The large alveolar surface of 75 sqm, and the minimal 4-8 μm barrier between alveoli air surface and capillaries are an ideal construction for gas exchange, but is vulnerable to inflammatory processes disrupting that barrier. While local and systemic inflammation is a normal and adequate response to various insults, the failure to contain inflammation is not. ARDS can, like septic shock, be understood as a failure of “the checks and balances” of the innate immune response. The risk posed by inflammation was demonstrated in a transgenic mouse model where activation of NF- κ B in lung epithelial cells was sufficient to cause neutrophil recruitment, pulmonary oedema, arterial hypoxemia, and cell death in the absence of any exogenous stimuli (95). Inflammation can be triggered by processes in the lungs as in pneumonia, aspiration, near-drowning or inhalation of toxic gases, induced by infections outside the lungs, or by non-infective causes, as pancreatitis, severe burns and multiple trauma. In ARDS caused by extra pulmonary insults, the inflammatory mediators, cytokines and activated leukocytes,

are carried by the blood to the lungs where they exert their effect (94;96). The course of ARDS can be divided into 3 phases, an acute exudative phase with activation and infiltration of leukocytes, increased permeability of the capillary-endothelial barrier, with leakage of protein rich fluids into the alveoli (97), this increased permeability oedema being a hallmark of ARDS (94). A fibro proliferative phase, occurring on the second to seventh day after the initiating injury follows, where fibroblasts infiltrate the site of inflammation and remodelling takes place, in most cases leading to full restitution, or contrarily, to progression to a fibrotic phase, with increasing fibrosis formation and loss of function (98). These phases have been confirmed in histopathological studies, and they correlate more with time than with the initiating insult (99).

Lately, the different elements of the inflammatory process, causing the clinical syndrome of ARDS, have been delineated, and details of the cytokine network, leukocytes and leukocytes derived inflammatory mediators elucidated (100). **Cytokines.** Tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are prototypically pro-inflammatory macrophage-derived cytokines, secreted shortly after insults like the injection of LPS or induction of pancreatitis (101), and in turn induce production of inflammatory cascades, inflammatory mediators, and up-regulate adhesion molecules on endothelial cells and leukocytes. In persistent ARDS, continuous high levels are found in BAL fluid (102). IL-6 is produced in response to TNF- α and IL-1 β stimulation, up-regulates the production of CRP, induces fever, and circulating levels correlate with severity of sepsis and pancreatitis-induced ARDS (103). IL-10 curbs inflammation by inhibiting secretion of pro-inflammatory cytokines and up-regulating production of their soluble receptors, such as IL-1ra (104). Low circulating levels correlate with poor prognosis. Transforming Growth Factor- β (TGF- β) is important in fibrosis formation in several disease conditions, but is also important in tissue injury resolution (105;106). In ARDS, focus has been on TGF- β involvement in lung fibrosis formation in the fibroproliferative phase (107). Recent studies suggest that it is also a key mediator of acute lung injury by increasing endothelial permeability (108). Other cytokines include granulocyte macrophage-colony stimulating factor (GM-CSF) a haematological growth factor, required for alveolar macrophage function, lung host defence, and surfactant homeostasis, where low levels have been associated with poor prognosis in sepsis (109). Chemokines, signalling molecules involved in attraction and activation of leukocytes to sites of inflammation, like the CC chemokines, IL-8, a potent leukocyte

attractant, and IP-10 a chemo attractant for Th1 cells activating cell-mediated immune response, produced by bronchial epithelial cells in response to infection, have both been shown to correlate with mortality in ARDS (110;111). The CXC chemokines, monocyte chemo attractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1 α , also play a role in ARDS, with increased levels shown to be associated with poor prognosis (112;113). In experimental models, neutralizing antibodies (114), or disruption of chemokine receptors have improved the outcome (115). The CXC chemokines have further been implicated in the residual pulmonary fibrosis developing during the fibroproliferative phase in a subset of ARDS patients (116). Future research is likely to identify other cytokines, as well as giving a better understanding of the complexity and redundancy of the cytokine network in ARDS.

Activated neutrophil granulocytes. The role of circulating and pulmonary polymorphonuclear leukocytes (PMN) as mediators of lung injury was recognized in the early 1980s, and is still central to the current concepts of ARDS. The detection of PMNs together with oedema fluid and hyaline membranes in autopsies (90), ultrastructural studies demonstrating the presence of increased numbers of intravascular and extravascular PMNs, platelets, fibrin, as well as both endothelial and epithelial injury, and additional observations of increased PMN numbers in BAL fluid (117), testified to the role of inflammation and PMNs in ARDS (118). Under physiological conditions, circulating neutrophils are in a resting state, and activation involves a series of co-ordinated events; the expression of cell-surface adhesion molecules (selectins and integrins), chemotaxis, the movement toward the site of inflammation through sensing of chemokine gradients, transendothelial migration and finally, phagocytosis and/or release of inflammatory mediators and oxygen radicals. In parallel, a simultaneous up-regulation of adhesion ligands takes place on endothelial cells at the site of inflammation (119). The concept of activation of PMNs, by cytokines, after direct pathogen contact, complement activation, or other insults, have thus been supplemented with the concept of a similar activation of endothelial cells in the lungs, induced in part by the same triggers (120). Interaction of activated circulating and pulmonary PMNs, activated pulmonary microvasculature, and the effect of other locally produced or circulating inflammatory signalling and mediator molecules represent currently the best model for the pathophysiology of ARDS (119). The PMNs effector molecules, producing damage to the lungs are numerous. The “respiratory burst” producing oxygen radicals within the phagolysosome of PMNs, or released extracellularly in a controlled manner in an adequate inflammatory response, could in an exaggerated

response be released in quantities provoking tissue damage (121). This excessive production of oxygen radicals could be further enhanced by delivery of high oxygen concentrations during ventilator support (122). PMNs contain a large array of potential tissue damaging enzymes like elastases, collagenases, matrix metalloproteinases, gelatinases, released in an uncontrolled manner and without a counterbalanced secretion of their inhibitors, severe tissue injury could ensue (123;124). Finally, uncontrolled systemic complement activation can contribute to lung tissue injury, in part being central to activation and recruitment of PMN's (125-127). This picture is simplified and other cells like circulating and pulmonary macrophages, and the lung parenchymal cells themselves almost certainly play a role in ARDS, as can be inferred by the fact that also profound neutropenic patients can develop ARDS (128). Knowledge of causes and consequences, and the basis of the immune dysregulation in ARDS on cytokine and cellular level, is poorly understood and a major obstacle to development of new interventions.

Several components of ongoing inflammation like cytokine production, cell-proliferation and remodelling are responsive to glucocorticoid treatment, and thus several intervention studies in ARDS have been conducted. The failure of glucocorticoid therapy in reducing mortality, although decreasing ventilator and ICU days, was shown in a large randomized study (129). Reduced mortality with early and prolonged treatment (Two weeks at full dose, then tapered off > 2 weeks) with low-dose glucocorticoids (Methylprednisolon 1 mg/kg per day for more than two weeks) was, however, reported in a successive multicenter study (130). Pooled data from four multicenter studies accounting for 472 patients showed that the relative risk of short-term mortality was 0,81 (CI 0,66-0,99) in favour of treatment with glucocorticoids (131). Differences may be related to timing of treatment initiation, dosage and duration, as well as heterogeneity of patients (132). Future research to improve ARDS outcome will imply an approach to elucidate details of cellular and molecular pathophysiology, as well as intervention studies that are both large enough, and account for the dynamic nature of ARDS, where therapies may be effective at certain time points but not in others.

1.5 Pulmonary infections and lung pathology caused by Influenza virus

Waterfowls and shorebirds are the natural reservoir of all subtypes of influenza A viruses, but the viruses can be transmitted to poultry, and, for some of the subtypes, to mammal species, including swine and humans. When sustained human to human transmission occurs, the viruses may cause pandemics, which upon adaptation to their human host, give rise to seasonal influenza outbreaks. A major factor for host specificity is receptor recognition, with avian viruses recognizing alpha 2,3 linked cellular receptors, present mainly in the lower respiratory tract of humans, while human-adapted influenza viruses recognize alpha 2,6 linked cellular receptors, more widely distributed in the human upper respiratory tract, supporting active replication and transmission of the virus (133). The recent pdm H1N1 pandemic proved to give rise to mild disease, with notable exceptions, as in patients presented in the last paper of this thesis (134). At the outset of the pandemic, however, widespread fear prevailed about the possibility of large numbers of patients developing respiratory failure due to influenza, possibly overwhelming health care resources. Considering the high mortality observed in the highly pathogenic H5N1 influenza virus that emerged in 1997, and that the exact reason for the extreme number of deaths in the 1918 “Spanish flu” is largely unknown or at least intensely debated, these fears are not unfounded. The 1918 virus has been reconstructed using reverse genetics. In both mouse and maquette models the reconstructed virus was shown to propagate very efficiently, giving rise to a far higher number of viral copies than seasonal flu virus, as well as inducing a “cytokine storm”, possibly triggering uncontrolled and destructive inflammatory host response leading to respiratory failure (135;136). This has also been demonstrated in H5N1 infections, and proposed as a reason for its high mortality (137). The 1918 H1N1 virus, however, did not harbour alterations in the HA cleaving site, and even displaying a highly pathogenic phenotype, with trypsin-independent replication, did not display Δ 146 mutations, nor did it have a NS1 mutation, enhancing anti-interferon activity (138), three features otherwise proposed to explain the virulence of highly pathogenic H5N1 influenza virus. Cleaving site alteration allows HA cleavage by a wide range of host proteases, not only confined to respiratory mucosal cells thus increasing organ tropism, and Δ 146 mutations have also been shown to alter cell tropism. Testing of single-gene reassortant 1918 viruses identified the crucial role of HA, NA and PB1 segments in virus replication

and virulence (139). In one study, however, where the authors gathered and analysed the extraordinary amount of contemporary data and studies generated during the 1918 pandemic, including surviving histopathological samples, another conclusion was drawn: secondary bacterial infections was the main reason for the high death toll (140). The specific inflammatory patterns or regulation of immune response and the pathogenesis of the cytopathic effects of influenza in humans are incompletely understood. Humoral immunity with neutralising antibodies is important for preventing viral spread, its production is the goal of vaccines, and specific antibodies can abort or limit disease. Non-specific immunity is however of major importance and PMNs and alveolar macrophages are important for phagocytosis of influenza virions (141). The constitutional symptoms of influenza are explained by the release of various cytokines produced in the airway mucosa and release systemically, cytotoxic CD8⁺ cell response to infected cells, as well as natural killer cells induced apoptosis are important in clearing infected cells (142). Influenza gives rise to a variety of clinical pictures, ranging from mild cold symptoms to tracheobronchitis with pronounced constitutional symptoms, but severe alveolar inflammation, presenting as primary viral pneumonia, is rare (143). Seasonal influenza binds predominantly to α 2,6-linked cellular receptors which in humans are dominant in the upper airways, whereas the pdm (H1N1) virus also binds well to the α 2,3 gal receptors, which are present in the conjunctivae, distal airways, and alveolar pneumocytes, and may explain the ability of the virus to cause severe viral pneumonia (144). The D222G/N mutation in pdm (H1N1), seems to confer even higher affinity to α 2,3 receptors, further increasing the risk of viral pneumonia. (145). The vast majority of patients infected with influenza virus experience mild upper respiratory tract disease or tracheobronchitis, but denuding of bronchial mucosa and impaired mucociliary clearance predisposes to secondary bacterial pneumonias (146). Frail patients may not tolerate this, but a major concern in influenza is the possibility of inducing severe viral pneumonia and respiratory failure in otherwise healthy patients (143). During the pdm H1N1 pandemic, viral pneumonia reemerged as an important cause of ARDS (147). The role of specific host factors, viral mutants, or the interaction of both in severe pdm (H1N1) is poorly understood. Both a cytopathic effect as well as induction of an overshooting immune response could account for the lung pathology findings, and an increased viral replicative capability would enhance both (148). In patients dying from pdm(H1N1) infection, the histopathological findings showed varying degrees of diffuse alveolar damage with hyaline membranes and septal oedema, vascular congestion,

tracheitis, and necrotizing bronchiolitis. Bronchopneumonia and evidence of secondary bacterial pneumonia were found in 26% to 38% of cases (149;150). With the great importance of influenza to public health, further research to understand the pathology of influenza remains a priority.

2. Aims of study

Paper one

To investigate the evolution of a local *Pseudomonas aeruginosa* outbreak caused by contaminated oral swabs in an ICU, and the impact on mortality and morbidity for mechanically ventilated patients being colonized in the airways with this pathogen.

Paper two

To investigate the patterns and dynamics of the microbial flora of the airways in mechanically ventilated patients, the correlation of findings over time and airway locations, the effect of antibiotic therapy on recovery rates, and the correlation between serial chest X-rays findings and bacterial counts in the distal airways.

Paper three

To investigate whether N terminal-pro atrial natriuretic peptide (NT-proANP) and N terminal pro brain natriuretic peptide (NT-proBNP) provide independent prognostic information in a consecutive series of critically ill patients requiring mechanical ventilation, admitted in the ICU for mainly non-cardiovascular diagnosis. To compare their relative prognostic value and see if they provide complementary prognostic information to conventional risk factors, including the SAPS II score, SOFA score and McCabe score.

Paper four

Investigate the immune response and role of viral factors in patients developing severe respiratory failure requiring mechanical ventilator support, caused by the new pdm (H1N1) influenza virus during the 2009 pandemic.

3. Summary of results

Paper one

Over the 5 month study period, defined as date of the first identification of the outbreak strain till last identified, 94 patients were admitted to the ICU. Forty-four were eligible for inclusion, fulfilling the criteria of at least 24 hours of mechanical ventilation and microbiological samples being collected. Eighteen (41%) of the patients became colonized with *P. aeruginosa*. Colonization occurred early with a median time to colonization of 4 days, and 14 out of 18 patients became colonized within 9 days. Seven of the colonized patients became infected with *P. aeruginosa*; 4 developed VAP, 1 bloodstream infection, and 2 intra-abdominal infections. Disease severity on admission day measured with SAPS II score was similar in colonized and non-colonised patients. SOFA score, a marker of response to therapy, improved slower in the colonized patients. Mortality was significantly higher in colonized patients, 10 vs 5 deaths, but so was underlying morbidity with 6 vs 1 patient in the McCabe class C, and 7 vs 18 in the McCabe class A group. Analysing mortality irrespective of colonization status, mortality was 57 % in the McCabe class C and 16 % in the class A group, almost identical to mortality analyzed according to colonization status. Colonization with *P. aeruginosa* was thus associated with higher mortality, but a causal effect could not be inferred from this study.

Paper two

In a cohort of 74 consecutively recruited mechanically ventilated patients, airway samples were collected from the oropharynx, the trachea and the distal airways (BAL fluid), 48 hours after admission to the ICU, and thereafter every 48 hours or until extubated. A second sample set was obtained in 47, a third in 28, a fourth in 16, and a fifth in 13 patients, respectively. Potential VAP pathogens were identified, quantified, and genotyped using standard techniques. Microbial findings were highly correlated both between airway locations and over time when samples were taken no more than 72 h apart. If no VAP pathogen was present in the oral flora, it was unlikely to find it in the lower airway sample. The positive predictive value of the oropharyngeal sample was 0.73 (95% CI 0.67–0.80), and the negative predictive value was 0.95 (95% CI 0.92–0.99). Colonisation with Enterobacteriaceae, nonfermentative bacteria and *S. aureus* was monoclonal in the airways

and over time, whereas colonisation with microbes normally found in the oropharynx, i.e., *H. influenzae*, and *S. pneumoniae*, was polyclonal. When antibiotics were used, the chance of recovering VAP pathogens from all sampling sites was reduced three-fold. No correlation was observed between a bacterial count of $\geq 10^4$ cfu /ml in BAL fluid and chest X-rays compatible with VAP.

Paper three

Seventy patients were included, 59 with non-cardiovascular diagnosis. Infection was the most common diagnosis group (n=25), followed by primarily respiratory problems (n=14), gastrointestinal problems (n=8), trauma (n=3) and miscellaneous (n=9). Twenty-five patients died. Median NT-proBNP level in serum 48 h after admission to the ICU was 154 (range 3–5895) pmol/l, median NT-proANP level was 1180 (range 283–7020) pmol/l (n =65). NT-proBNP and NT-proANP levels were significantly higher in non-survivors than in 30-day survivors, median (25th–75th percentile): NT-proBNP 438 (282–1356) vs. 59 (20–368) pmol/l, respectively (P= 0.001), and for NT-proANP 1845 (1190–2780) vs. 993 (692–1890) pmol/l (P= 0.002). Patients with supramedian NT-proBNP and NT-proANP levels had a higher mortality rate than those with inframedian values, odds ratio (OR) 5.74 (95% CI 1.91–17.28, P 5 0.002) and 5.74 (95% CI 1.77–18.55, P=0.004). The relative prognostic value by the AUC (c-index) was 0,68 for SAPS, 0,74 for log NT-proBNP, 0,73 for log NT-proANP, increasing to 0, 76 and 0, 75 respectively when combined with SAPS. Thus, NT-proANP and NT-proBNP levels were both associated with decreased short-term survival in unselected, mechanically ventilated ICU patients. NT-proANP performed equally well as a prognostic indicator as NT-proBNP and may represent an alternative to NT-proBNP. Prospective studies involving a larger number of patients will be needed to determine the clinical utility of NT-proANP and NT-proBNP in this setting and to define prognostic cut-off points.

Paper four

Seven patients with severe pdm (H1N1) influenza defined by positive airways pdm (H1N1) polymerase chain reaction (PCR), bi-lateral diffuse lung chest X-rays infiltrates, need for mechanical ventilator support, but without significant co-morbidities, were compared to 7 age and gender matched healthy controls. Blood samples were collected at inclusion, at day 3 and 6. Four patients were viremic at inclusion, and in two, in subsequent samples. In two

patients with viral loads above 1000 genomes/ml, mutant D222G/N pdmH1N1 virus was identified. With micro array analyses, compared to healthy controls and false discovery rate (FDR) < 5%), 370 genes were found at least 2-fold up-regulated (N= 259) or at least 50% down-regulated (N= 111) in whole blood samples from study inclusion. Among the top up-regulated genes, several were linked to neutrophil differentiation and activation, or encoding neutrophil granule proteins like CD177, MMP-8, S100A12, S100A9 and ANXA3. This up-regulation continued over time so that on the last sampling day, 14 of the 30 genes with the highest fold change, coded for products located in neutrophil secretory granules, linked to granulocyte maturation, or having known antibacterial activity. Activation was also seen at the protein level, reflected by a marked increase in plasma levels of the granulocyte-derived enzymes elastase and lactoferrin persisting throughout the observation period. Among down-regulated genes, natural killer cell associated genes (e.g., KLRG1, KLRD1 and CD244), genes encoding lymphocyte surface markers (CD4, CD2, CD1c, C3XCR1), and genes involved in T cell activation like NFATC2 were identified. Genes encoding antigen presenting molecules of MHC II class were consistently down-regulated. Notably, no genes encoding inflammatory cytokines or interferons were up-regulated. However, several cytokine response genes like IFN- α response gene IFI27, and TNF and IL-18 receptor genes were up-regulated. Markedly activation of the whole complement cascade was demonstrated by high levels of the terminal complement complex (TCC), the elevation being highest in the sickest patients. In contrast to the finding on mRNA level in whole blood, 16 of the 29 tested cytokines, including interferons, interleukins, chemokines and growth factors, were significantly elevated in patients at study inclusion versus controls.

Levels of IFN- α , as well as the prototypical pro-inflammatory cytokines TNF- α and IL-6, Th1 (i.e., IFN- γ), Th17 (i.e., IL-17) and Th2 (i.e., IL-9) derived cytokines, were elevated, as well as several chemokines, including both CC chemokines (i.e. MCP-1 and eotaxin) and CXC chemokines (i.e. IL-8 and IP-10), with particularly high levels of IP-10 (>100-fold increase). In addition to pro-inflammatory cytokine increase, the patients showed markedly raised levels of the anti-inflammatory mediators IL-10 and IL-1Ra (>10-fold). Most of the changes in cytokines persisted throughout the observation period. Growth factors involved in matrix remodelling, fibrosis and angiogenesis (i.e. FGF, PDGF and VEGF), were also elevated with particularly high levels of PDGF (>50-fold). The increased mRNA level of MMP-8 in blood was also seen at the protein level in plasma with markedly increased

MMP-8 in the patients (>10-fold). This MMP-8 increase was accompanied by a significant increase in several endogenous tissue inhibitors of MMPs (i.e. TIMP-1, TIMP-3 and TIMP-3), further underscoring an ongoing process of matrix remodelling in these patients. In contrast to MMP-8, no increase in MMP-9, and MMP-12 was found the latter two down-regulated in the patient group, suggesting that the MMPs were differently affected by severe H1N1 influenza infection.

4. General discussion

4.1 Paper one

P. aeruginosa is among the most frequently isolated pathogens from airway samples of patients on invasive mechanical ventilation (151;152). *P. aeruginosa* is also consistently reported in the context of outbreaks (153). Organisms within the *Pseudomonas* genus are highly versatile, adapt to a wide range of habitats, even growing in distilled water, accounting for their constant presence in the environment (153). The prevalence of colonization in healthy persons outside of hospitals is low (154). In hospitalized patients carriage is increased, and is associated to frailty, mechanical ventilation, oral feeding tubes and use of antibiotics (155-157). The source and transmission routes of colonization are often difficult to trace, in outbreaks however, contaminated hospital equipment is often implicated (153). The first aim of the presented study was to describe an outbreak effectively propagated by contaminated oral swabs. This outbreak was part of the largest ever *P. aeruginosa* outbreak in Norway, the contaminated swabs being distributed nationwide (158). At our institution colonization occurred early, half of the patients being colonized within 4 days after admittance to the ICU; it was widespread with a 41% colonization rate, thus reflecting a highly efficacious direct oropharyngeal inoculation route. The outbreak was the second with *P. aeruginosa* in our ICU, the first being caused by contaminated tap water and successfully stopped by increasing water temperature in boilers, pipes and taps, along with strict barrier nursing methods (159). In the inter outbreak period, where a comprehensive *P. aeruginosa* screening was in place, the recovery rate of *P. aeruginosa* was less than half the rate during the outbreak, and isolates were polyclonal, testifying to effective cross-contamination precautions, and to the propensity of *P. aeruginosa* to occur in outbreaks, at least in our ICU. The second aim of the study was to investigate the impact of colonization on patient morbidity and mortality. *P. aeruginosa* does not, with few exceptions, such as after inoculation in traumatized skin or ear canal,

cause disease in normal hosts (153). *P. aeruginosa* possesses several virulence factors (160), and trauma to the mucosa, pooling of contaminated oropharyngeal secretions, and creation of a foreign body surface by the endotracheal tube, can all contribute to a transition from colonization to infection (66;70). Distinguishing colonization from infection is not straightforward in ventilated patients. Therefore, two investigators separately reviewed and scrutinised each patient file before categorizing to either of them. When there was discrepancy, a consensus was reached after a re-review. Seven of the 18 colonized patients, or roughly a third, were considered to be infected. This is lower than in the study presenting this outbreak on a national level (158), where 161 out of 231 patients, or roughly two-thirds, were considered infected. Though relatively more septicaemias were found, the high number of pneumonias explain this difference, confirming the difficulty of separating airway colonization from pneumonia, and the subjective nature of this distinction, at least in ventilated patients. We found a significantly higher mortality in colonized vs. non-colonized patients, 55%, vs. 19%. The two groups were similar for most demographic variables, regarding conditions leading to ICU admission, and SAPS II score reflecting disease severity at admission. They differed, however, regarding co-morbidity and response to therapy. McCabe classification, a marker of co-morbidity was as measure of the former, and the rate of decline of the SOFA score for the latter. The death rate in colonized and infected patients was similar, but the numbers were too low to warrant formal statistical analysis. Colonization was probably not a causal factor for mortality. A similar difficulty, delineating cause and association, was also seen in the outbreak investigation on the national level. Investigators found underlying disorder to be of major importance, with all 71 deaths occurring in patients with severe underlying diseases. They went, however, further in their conclusion, stating that in 31 of 71 patients *P. aeruginosa* infection contributed to death, in 21 probably not, and in the rest uncertain or not possible to evaluate. Support in the literature can be found for either view, colonization with large microbial burdens being an independent risk factor for death (161), or colonization per se having no attributable mortality, only if leading to VAP (162). A major methodological difficulty in outcome studies with this and similar opportunistic pathogens like *Stenotrophomonas* and *Acinetobacter*, is that risk of acquisition, risk of infection, and risk of death from underlying disease covariate, often making it impossible at both single patient level, and in cohorts to establish the ultimate cause of death.

4.2 Paper two

In this study of scheduled microbial samplings from three airway locations in long-term ventilated patients, several observations were made. By genotyping all isolates considered to be potential VAP pathogens, it was demonstrated that they were monoclonal over locations and time, implicating that once established colonizing bacteria occupy an ecological niche, and there is no constant turnaround of microbes seeding the airways from the environment or from other locations in the patient. This applies at least over a period of 72 hours, where correlations were found to be between 0, 71 and 0, 88. Within 17 patients identical bacterial clones were found more than 6 days apart, and observation over this time interval was possible for 28 patients. The high proportion of patients keeping the same clone over this prolonged period, confirms the stability of the established flora. These observations are in line with the results from the largest study ever conducted to assess the relevance of separating early VAP from late VAP (83). Although having a different design, comparing the distribution of pathogens recovered in patients with early and late onset VAP (early < 7 days, and late >7 days of invasive mechanical ventilation), in more than 16 000 VAP cases, they found no difference pointing to an evolution towards more MDR bacteria over time. We found the distribution of potential VAP pathogens nearly identical in samples collected 2 days and 11-14 days after initiation of ventilation, with exceptions for *S. aureus* which increased in late BAL samples and *Candida* spp which increased in late oropharyngeal samples, but numbers of patients are small. Genotyping showed that bacteria normally found in the oropharynx, such as *S. pneumoniae*, *H. influenza* and *H. parainfluenza* were polyclonal, possibly implicating that monoclonality is observed only with recently introduced bacteria, such as Enterobacteriaceae in ventilated patients, but again small number of patients preclude too firm conclusions. The observation in this study of an equally high correlation of possible VAP pathogens in three sampled locations, oropharynx, trachea and BAL fluid, was recently reproduced in a study with a similar design.(163). Here, the authors found that isolates of *S. aureus*, *P. aeruginosa*, *Acinetobacter* species, and enteric species recovered from dental plaque from most patients were indistinguishable from isolates recovered from BAL fluid, and concluded that “respiratory pathogens isolated from the lung are often genetically indistinguishable from strains of the same species isolated from the oral cavity in patients who receive mechanical ventilation”. Our suggestion, to use results of oropharyngeal surveillance cultures to guide therapy in suspected VAP, was recently evaluated in a large study (164). Out of VAP 136 patients with a positive culture

collected with a plugged distal bronchial sampling procedure 125 had a positive culture at ICU admission. The correlation between these two specimens was 85 %, translating into a likelihood ratio > 6 , a threshold considered significant to accept the diagnosis. In this study VAP diagnosis and sampling was done within 5 days of ICU admission. In another study, endotracheal surveillance cultures were collected twice weekly and proved predictive of pathogens sampled when VAP was diagnosed, with 83% correlation between the two (165). Based on the high correlation between oropharyngeal and endotracheal cultures found in the presented study, one might suggest that the even more easily collected oropharyngeal samples could be used. Empiric VAP therapy guided by previous surveillance samples is still controversial, and Hayon et al did not recommend this strategy due to lack of correlation between surveillance and VAP cultures (166). In their study, however, mean time from surveillance culture to VAP was 8 days, and cultures collected from locations outside the respiratory tract were also considered as surveillance cultures. Thus, surveillance has to be performed twice weekly, and only airway samples should be considered relevant for surveillance related to VAP diagnostics. Finally, we assessed correlations between bacterial counts accepted as thresholds for VAP in distal airway samples and chest X-rays suggesting VAP were performed. No such correlation was found, and the lack of correlation was not explained by differences in antibiotic usage, as the proportions of patients receiving antibiotics and had growth of $\geq 10^4$ CFU/mL of a VAP pathogen were the same for patients with and without infiltrates (nine of 18 and 12 of 20, respectively). The strength of this analysis was the ability to investigate serial X-rays in a majority of the patients, excluding VAP diagnosis where chest X-ray changes were transient. Most VAP studies use single X rays to establish the diagnosis of VAP, but it is well recognized that chest X rays infiltrates are both common and caused by several conditions other than VAP in ventilated patients, and infiltrates caused by pneumonia only resolve over weeks.

4.3 Paper three

Several prognostic markers have been investigated in different ICU patient populations, including patients with VAP and sepsis. Markers of inflammation such as C-reactive protein and pro-calcitonin and cytokines (regulating their production) (167-169), as well as markers such as cholesterol and thyroid hormones have been evaluated (170;171). The natriuretic peptides are a family of structurally related hormones, released from the atria (ANP) or ventricles (BNP and ANP) in response to stretch, and thus markers of cardiac wall distension (172). In cardiology they have been firmly established as markers of heart failure, but also proven their value in other cardiovascular diseases, including congestive heart failure, acute coronary syndromes and valvular heart disease (173;174) Both ANP and BNP are cleaved upon secretion and their biologically inactive N-terminal end, NT-proBNP and NT-proANP are more stable, and preferred for routine measurements. The relative prognostic merit of these two markers has been compared in several cardiac conditions such as after myocardial infarction, aortic stenosis, and hypertrophic cardiomyopathy (174-176). In critically ill patients, their role has been more controversial, and notably in sepsis and septic shock association between levels of these peptides and ventricular filling pressure have been weak (177). Several mechanisms could contribute to the release of natriuretic peptides in the critically ill, including right ventricular overload, catecholamine therapy, renal failure, diseases of the CNS, and cytokine up-regulation (178). In a recent review, an excellent prognostic value of natriuretic peptides in ICU patients was reaffirmed (179). There are few reports of the relative performance of NT-proBNP and NT-proANP in ventilated ICU patients, and they have been reported to be differentially secreted in critically ill patients (180). In the presented study we compared their performance as prognostic markers in the same population of unselected mechanically ventilated patients. This cohort was the same as the one presented in paper two of this thesis, and comprised patients admitted to the ICU for mainly non-cardiac conditions, the majority admitted due to infections and lung diseases. Blood samples for NT-proBNP and NT-pro ANP measurements were collected 48 hours after ICU admission, as this sampling time represented the first sampling in the airways colonization study, from which these patients were recruited. This delayed sampling may explain why the levels were lower than in another study with similar design, where sampling was performed at ICU admission (181). In a large Finnish study of patients with sepsis and septic shock, NT-proBNP sampled on day three after admission, but not at admission, was an independent predictor of mortality, thus optimal sampling time for

prognostication need to be defined, and may differ for various patient categories (182). Although prognostic value of NT-proBNP has been investigated in several ICU patient populations, including unselected ICU patients, less is known about NT-proANP (181;183;184). A previous study showed levels to be higher in non-survivors than survivors with septic shock (185). The novelty of this study was by means of multivariate regression analysis to show that also NT-proANP is an independent prognostic marker, performing equally well as compared with NT-proBNP in unselected mechanically ventilated patients. The value of NT-proANP as prognostic marker was recently also investigated in lower airways infections (186), but the focus of recent research in natriuretic peptides has shifted to NT-proBNP. In the presented study both peptides were significantly higher in non-survivors than survivors, and in the entire cohort mortality was higher above than underneath the median value. In univariate analysis increase of 1 standard deviation increased the odds ratio for 30 days mortality with 2,55 (log NT-proBNP) and 2,48 (log NT-proANP), and the relative prognostic value by the AUC (c-index) was higher than for the SAPS II score (0,74 and 0,73 vs. 0,68), and in a combined model with SAPS II marginally additive (0,76). A recently published large Finnish multicenter study in unselected mechanically ventilated patients including 958 patients also found higher values in 90 days non-survivors. The baseline NT-proBNP AUC predicting 90 days mortality was similar to ours, 0,718. In this large sample they also determined a baseline cut-off value of 1765 pg/ml independently associated with mortality. Still, the authors could not recommend NT-proBNP for routine prognostic purpose as it did not add any value to other clinical data in patients with respiratory failure (187). Thus the role for these markers is still unclear, although we would agree with the summary remarks in a recent review of cardiac biomarkers in the critically ill: "First, specific assays have limitations, assay cut-offs have not been clearly established, and measurements during dynamic critical illness may be problematic. Second, biomarker interpretation may vary depending on individual patient characteristics and ICU diagnosis. Last, any marker measured in isolation is unlikely to surpass careful bedside assessment" (188).

4.4 Paper four

The 2009 pdm(H1N1) pandemic was mild in terms of overall mortality, with death rates not significantly higher than during seasonal epidemics (189). There was, however, a marked difference in age-specific mortality rates, with a higher mortality rate in the young without serious underlying co-morbidity, and a lower mortality than for seasonal influenza epidemic in the elderly, a situation reminiscent of the 1918 pandemic (189). A foreboding of such a scenario came from reports in the southern hemisphere predating the pandemic on the northern by a few months (190), enabling us to design a prospective study targeting patients with serious respiratory failure, investigating putative viral factors and host responses explaining the serious outcome of pdm(H1N1) infection. The pathogenesis of the lower respiratory tract pdm(H1N1) is poorly understood. The reconstructed 1918 H1N1 virus and the highly pathogenic H5N1 virus have a different genetic basis for their virulence, but share a phenotype of highly effective replicative capacity, and the induction of a strong cytokine production ("cytokine storm") (191). Viral load at the site of infection is thus important for subsequent inflammatory and tissue damaging response. This was recently demonstrated for pdm(H1N1) infection in a macaque model, where immunohistochemistry showed that, compared to seasonal influenza, pdm(H1N1) virus caused significantly more pulmonary lesions, correlated to more abundant virus in the lower respiratory tract. (192). Interestingly, the inflammatory response was not qualitatively but quantitatively different, gene expression profiles from lung tissue in seasonal and pdm(H1N1) being similar. A correlation between pharyngeal viral load and cytokine production was also recently published in a clinical study of severe pdm(H1N1) infection (193). In the presented study, 4 out of 7 patients had viremia, which is otherwise rare in seasonal influenza. It is conceivable that the observed viremia reflect both a large viral load in the lungs, indicative of effective replicative capacity, and a disruption of the alveolar capillary integrity caused by ensuing inflammation. Secondly, two out of four viremic patients displayed the D222G/N mutation as the viremic strain, as opposed to 222D in their airway samples. One of the remaining two had a 222D in both airway and serum, while viral load was too low to allow sequencing for the last viremic patient. The finding of D222G/N mutant lends further credit to viral factors as important in determining outcome in severe pdm(H1N1) influenza. In a recent study, viremia was found to be strongly associated with severe outcome in pdm(H1N1) and was detected in 14 out of 139 hospitalized patients, the D222G/N quasispecies detected in 90% of the viremic patients and thus strongly correlated to viremia (193). The capacity for

increased binding of the D222G/N mutant to cells of the lower respiratory tract, was recently demonstrated in a study where the D222G/N mutation was introduced in a prototype pdmH1N1 by reverse genetics, and the effect on virus receptor binding, replication, antigenic properties, and pathogenesis and transmission in animal models was investigated (194). Here, increased binding to macrophages and type II pneumocytes in the alveoli, and to tracheal and bronchial submucosal glands was demonstrated. This observation could explain why we did not find peripheral blood gene expression of up-regulated pro-inflammatory cytokines, but elevated cytokine levels in peripheral blood, the site of production possibly being infected pulmonary macrophages and type II pneumocytes. These findings were recently replicated in a study showing up-regulation of pro-inflammatory cytokines in lung tissue, but down-regulated in peripheral blood leukocytes, in patients dying of pdm(H1N1) influenza (195). Further support for pulmonary and not peripheral blood production of pro-inflammatory cytokines are reports of higher TNF and IL-1 concentrations in BAL fluid than in serum in patients with ARDS, suggesting pulmonary production (102;196). The histopathological findings in severe pdm(H1N1) infections are diffuse alveolar damage (DAD) with alveolar hyaline membranes, the histopathological correlate of ARDS (197;198). In viral pneumonia, the histopathological changes are interstitial lymphocytic infiltrate without alveolar exudates. The line between severe viral pneumonia and ARDS is blurred and more semantic than biologic, the former being etiologically defined and the latter as a clinical syndrome. Infections are, however, recognized as a major cause of ARDS, and in the context of severe pdm(H1N1) infection, where a longer and more severe immune response culminate in a more destructive viral infection, the term is appropriate. Patients in this study were too unstable to tolerate sampling from the lungs, the primary site of inflammation. We thus had to resort to blood sampling for investigation of the host immune response. Blood represents both a reservoir and a migration compartment for cells exposed to infectious agents and taking part in inflammatory processes, and constitutes an accessible source of information representing host immune response (43). Micro-array analyses identified 370 genes at least 2 fold up or 50% down-regulated compared to healthy controls, with a FDR < 5%. The analyses were repeated and results confirmed by real time quantitative PCR (RT-qPCR) for selected genes. A criticism of this study is the lack of control group with less severe disease, making a quantitative analysis of the immune response in severe and less severe disease difficult. The micro-array analysis using a healthy control group had the advantage of making a

qualitative analysis of involved immune response genes easier. Gene-expression profile in healthy controls were markedly similar, thus a clear distinction could be made towards sick patients, with multiple testing of 22 000 genes and $FDR < 5\%$ still identifying 370 differentially expressed genes. In a second set of analysis examining gene expression in patients on the first and last day of sampling, expression diversity was too great to allow application of the $FDR < 5\%$ criteria, as no differentially expressed genes would have been discovered. Though eliminating false positive gene-expression differences, an unacceptably high rate of false negative would have been created. A similar situation regarding micro-array analysis could be envisaged comparing severe and less severe pdm(H1N1) patients, given the limited number of patients that could be recruited during the short pandemic peak. Considering the dynamic nature of the immune response, temporal differences in sampling are of major importance. This study was not primarily a micro-array study, and the full spectrum of possible micro-array analysis was not performed. The Ingenuity® analysis tool was used for analysing gene networks and pathways, identifying inflammatory response, infectious disease and pulmonary disease as the categories with the most up-regulated genes. Based on findings in several earlier publications, a hypothesis-driven analysis was also performed, suggesting a prominent role of neutrophils in the pathogenesis of ARDS (199;200). The observation of several genes involved in neutrophil differentiation, activation, or encoding neutrophil granules, this gene-expression profile increasing over the observation period leading to 14 of the 30 most up-regulated genes belonging to this category on the last sampling day, strengthen the hypothesis. A pattern of down regulation of T-cell surface markers, like CD4, CD2, CD1c and T-cell activation markers such as NFATC2 was also observed, as well as consistent down-regulation of MHC II class molecules, possibly pointing to defective antigen presentation and impairment of development of adaptive immunity in severe pdm(H1N1). This was also shown in a recent study with similar design, but with far more comprehensive and formal analysis of the micro-array data, suggesting impairment of adaptive immunity development allowing persistent viral replication and continued cytokine production (201) . Our findings of persistent viremia in two out of four patients and the persistent elevation of cytokines over the study period could strengthen this interpretation. The micro-array results were further confirmed on the protein level, demonstrating persistent high levels of the neutrophil mediators MMP8, elastase, and lactoferrin, the former two involved in tissue degradation and remodelling. The role of these proteases in the pathogenesis of neutrophil-mediated

lung injury and ARDS is increasingly recognized (202-204). In particular, the elastinolytic activity of elastase is recognized to be central to alveolar injury seen in acute and chronic lung disease (205;206). In influenza infections the complement system is activated via the classical pathway by binding to virus specific neutralizing antibodies. However, alternative activation pathways have been demonstrated, and complement activation via non-neutralizing natural antibodies (representing the spontaneous repertoire of circulating immunoglobulin's, i.e. not generated after pathogen exposure), has been shown to mediate influenza neutralization in a similar way (207). In the present study high levels of the terminal complement complex (TCC) demonstrate both a strong activation, and activation involving the whole complement cascade. Viremia could have contributed to the activation, and the host-damage capacity of unchecked complement activation could have contributed to the seriousness of the disease. The most pronounced activation was found in the two patients with mutant virus and the most serious clinical outcome, lending further credit to this notion. Complement activation is an upstream mediator in the immune system, leading to activation of granulocytes, monocytes and macrophages. An alternative or additional role of the complement activation seen in this study could have been enhancement of excessive cellular innate immune response. A broad panel of cytokines, chemokines and growth factors was also investigated. Sixteen out of 29 tested cytokines including interferons, interleukins, chemokines and growth factors were significantly up-regulated, and in this longitudinal study, elevations were consistent over the observation period of 6 days. The prototypical pro-inflammatory cytokines TNF- α and IL-6 were markedly up-regulated as well as the Th1 derived IFN- α , and Th17 derived IL-17 cytokines, and the anti-inflammatory mediators IL-10 and IL-1Ra reflecting a net inflammatory phenotype in severe pdm(H1N1). IL-8 is the major neutrophil chemoattractant to the lungs, and can be secreted in large quantities by lung fibroblasts and type II epithelial cells (208). IL-8 in BAL fluid has also been shown to be a potential marker of development of ARDS, and serum levels of both IL-8 and IL-6 have been shown to correlate with the severity of ARDS, and lately specifically with pdm(H1N1) (206;209;210). Particularly high levels of IP-10 (> 100 fold), a CXC chemokine causing migration and activation of T-cells and NK cells into inflamed tissue were seen. The highest levels were observed in the two sickest patients as for TCC, possibly pointing to excessive production contributing to non-resolving systemic and pulmonary inflammation. Markedly enhanced level of growth factors involved in matrix remodelling, fibrosis and angiogenesis (FGF, PDGF >50 fold, VEGF) together with the

elevated elastase and MMP8 levels could indicate an ongoing pulmonary remodelling characteristic of severe respiratory disease. Conflicting results regarding the cytokine/chemokine profile in severe pdm(H1N1) have been reported. Bermejo-Martin et al found a similar Th1 and Th 17 cytokine/chemokine up-regulation in severe infections with high TNF- α , INF- γ / IP-10, MCP-1, and IL-17, IL-8 and IL-6 levels, respectively (210). A strong Th 1, Th 17 response was also found by de Castro et al (211). A similar pattern was recently published in a study from Hong-Kong (212). High levels of IL-6 and TNF- α was confirmed while IP-10 level in contrast, down-regulated in another recent paper (213). The reason for this discrepancy for IP-10 is unclear.

In summary, severe outcome in pdm(H1N1) is correlated to viremia and the D222G/N mutant. The lung histopathology and clinical picture in severe disease is similar to ARDS of other causes, the immunological mechanisms responsible for tissue damage overlap, an impairment of adaptive immunity could explain continuous viremia and persisting immune stimulation, and the cytokine profile over several studies is broadly similar, displaying a Th1, Th17 response and high levels of IP-10.

5. Concluding remarks

In studying the airway microbiology of mechanically ventilated patients, we have shown that these patients are vulnerable to colonization with opportunistic bacteria from the environment, and contaminated medical equipment poses a particular threat. Colonization with *P. aeruginosa*, one of the most frequently isolated pathogens in the airways of ventilated patients, is associated with mortality, but in this study it remains unclear if the relation is causal, as underlying co-morbidity was more severe in colonized patients. The microbial flora which is established in the airways of ventilated patients is stable over time, and potential VAP pathogens recovered from the distal airways, are almost always present also in the oropharynx. A possible consequence of this finding is to perform surveillance sampling of the easily accessible oropharynx to guide antibiotic treatment of VAP. Correlation between findings of bacteria above the quantitative threshold considered diagnostic for VAP and chest X-ray compatible with pneumonia was poor, and this study challenges the utility of this criterion. NT-proBNP and NT-proANP were equal and strong prognostic markers in unselected mechanically ventilated patients, but whether they add clinical relevant information in prognostication need further investigation, and cut off levels

have to be defined in larger studies. A mutant pdm(H1N1) influenza virus, the D222G/N mutant, conferring higher virus binding to the lungs could be one reason for severe outcome of influenza infection. The immune response in severe influenza with respiratory failure is characterized by Th1, Th17 cytokine response and high levels of IP-10, a marked neutrophil and complement activation, and down regulation of T-cell markers, possibly pointing to impairment of adaptive immunity.

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