# Strategies to improve clinical management of community-acquired pneumonia

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# Strategies to improve clinical management of community-acquired pneumonia

## Strategieën om de behandeling van een buiten het ziekenhuis opgelopen longontsteking te verbeteren

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 9 februari 2012 des middags te 2.30 uur

door

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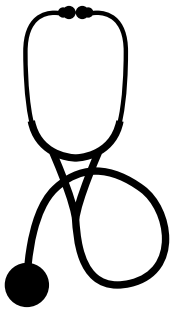

# Chapter 1

## General introduction and outline of the thesis

## Introduction

Pneumonia is an inflammation of the pulmonary parenchyma that is caused by infectious agents. The clinical syndrome is characterised by local inflammation of the lung, which is reflected by pulmonary symptoms, such as cough, sputum production, chest pain, and dyspnoea. The systemic inflammatory response results in a diversity of symptoms, such as fever or hypothermia, sweats, and chills. Chest radiograph may reveal pulmonary lesions, and laboratory results frequently show leukocytosis and an elevated C-reactive protein (CRP) (*Figure 1*). Pneumonia can be divided into community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP).[1-4] Community-acquired pneumonia is one of the most common infectious diseases requiring hospitalisation, and it is the third leading cause of death worldwide.[5] In the Netherlands, the overall incidence of pneumonia is high and lies around 8.3 per 1000 men and 8.4 per 1000 women. This incidence rate corresponds to an estimated 135,000 cases per year. The incidence is higher in young children and elderly (*Figure 2*). Of all episodes of CAP, an average of 22,425 patients are hospitalised and 5,500 patients die because of CAP each year (calculations based on the period of 2001-2006).[6]

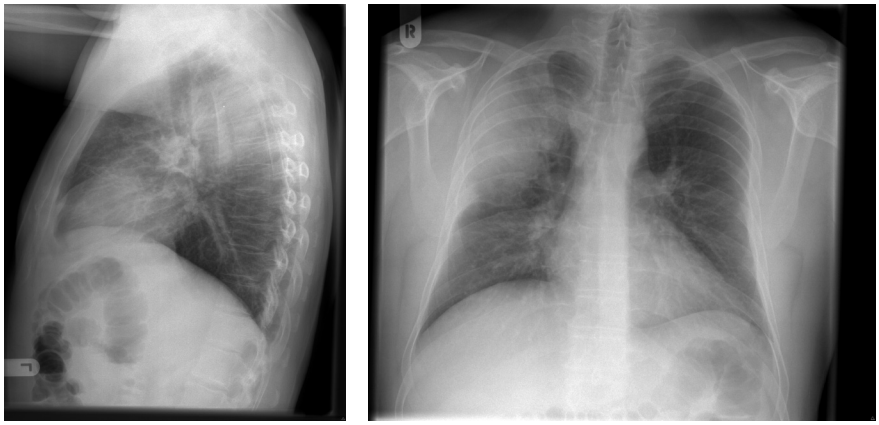

**Figure 1.** Chest radiograph

In non-immunocompromised patients, *Streptococcus pneumoniae* is the most frequently identified microorganism in CAP, followed by *Haemophilus influenzae*, *Legionella* species and viruses.[7-10] The management of pneumonia has been complicated in the last decades by expanding antimicrobial resistance, increasing age and the growth of the immunocompromised population.[11-13]

In addition to these difficulties, outbreaks of *Coxiella burnetii* pneumonia in 2009-2010 and Mexican fever in the autumn of 2010 complicate the management of CAP and result in significant healthcare costs.[14]
The mainstay of CAP therapy is early diagnosis and the initiation of appropriate antibiotic therapy within four hours to minimise the door-to-needle time (time to administration of antibiotics).[15] Despite advances in prevention by vaccination, microbiological diagnostics and antibiotic therapy, pneumonia is still characterised by a high mortality and morbidity and is associated with significant healthcare costs.[16,17]

In this thesis, we investigated strategies to improve the quality of clinical management in patients with pneumonia and to reduce healthcare burden.
First, we investigated the prevention of pneumonia. In that context, we studied the factors that are associated with an increased susceptibility for the development of CAP. Other preventive strategies, e.g. vaccination, are beyond the scope of this thesis. Second, we tried to improve the understanding of the immune response in CAP. A better understanding of the immune response might lead to prevention in patients with an increased susceptibility and in the future, to new treatment options. Furthermore, we explored the predictive value of possible biomarkers that can help predict the severity and outcome of CAP. Currently, treatment protocols are based on the pneumonia severity index or AMBU-65 score.[18,19] New biomarkers might improve the predictive values of these scoring systems. Last, we investigated adjunctive therapy besides antibiotics in patients with CAP.

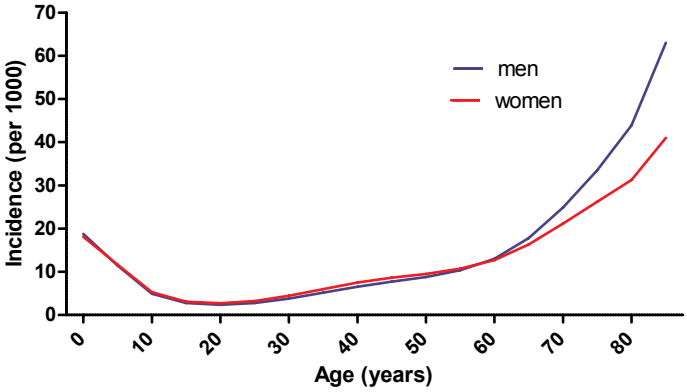


**Figure 2.** Incidence (per 1000) of pneumonia in the Netherlands in 2007 stratified to age and sex.
*(Adapted from National Kompas Volksgezondheid [20])*

**Susceptibility to and prevention of pneumonia**

In the beginning of the 20th century, better hygiene measures and the development of sanitary facilities reduced the incidence of infections, including CAP. Over the last several years, a reduction in the incidence of pneumonia has been achieved by vaccination.[21] It is likely that vaccination against H1N1 influenza has reduced the burden of pneumonia in the last two years.[22] Moreover, since April 2006, all Dutch children are vaccinated with a 7-valent pneumococcal conjugate vaccine. This vaccine is not suitable for prevention of CAP because it has a theoretical coverage of 31% of all invasive *S. pneumoniae* serotypes in adults.[23] The pneumococcal vaccine which is currently being tested for effectivity against CAP is a 13-valent pneumococcal conjugate vaccine which has a much higher coverage (61%).[24] For both vaccines, the actual percentage protection is often lower due to suboptimal immunogenicity in the at-risk groups. However, vaccination against *S. pneumoniae* is an important strategy in the prevention of pneumonia. Vaccines against non-encapsulated *H. influenzae* or *Legionella* species are not currently available. As illustrated by circumstances in 2010, other measures, such as fast interventions by the government to prevent the spread of an outbreak of disease (*Coxiella burnetii*) are important in CAP prevention.[25,26] Because the intensive animal husbandry farms still exists, rapid interventions to stop the spread of new animal-borne diseases are needed.

Recent literature suggests that certain medications may alter the risk of pneumonia. The use of angiotensin-converting enzyme (ACE) inhibitors and statins has been linked to a reduced risk of pneumonia[27], while gastric acid suppressing drugs and sedative medications are associated with an increased risk of CAP.[28-31] A better understanding of the mechanisms of these modified risks and the careful prescription of these medications might eventually lead to better strategies for pneumonia prevention.

**Immune response in community-acquired pneumonia**

The lung defends itself against a potentially hostile environment using three main mechanisms: mechanical defences, the innate immune system and the adaptive immune system. Mechanical defences include coordinated movement of cilia on the respiratory epithelium and cough and sneezing reflexes. The innate immune system includes both cellular (phagocytes) and humoral (complement) responses.[32] The adaptive immune response is characterised by two major systems, antibody- and cell-mediated immunity, which are generated by antigen specific B and T lymphocytes, respectively.[33] In this thesis, we will mainly focus on the role of the innate immune system.

**Recognition of microbes**

When the mechanical defences of the lung are unable to stop the microbial invasion of the alveolar space, the innate immune system is activated. Pathogen recognition receptors (PRRs), such as Toll-like receptors, recognise pathogen-associated molecular patterns (PAMPs), which are invariant structures of pathogens that are essential for microbial pathogenicity or survival.[34] Mannose binding lectin (MBL) and ficolin-2 (FCN2) are components of the complement system and as such constitute a separate PRR class (*Figure 3*).[35,36] These lectins bind to mannose residues that are abundant on the cell surface of most microbes. After binding, the formation of the membrane attack complex occurs, which results in the lysis of the microbe. Functional MBL levels are influenced by single-nucleotide polymorphisms (SNPs) in exon 1 and the MBL2 gene promoter region. The combination of these SNPs results in sufficient or deficient MBL serum levels.[37] Also, polymorphisms in the FCN2 gene have been described.[38,39] Genetic differences in the 5' untranslated region influence FCN2 levels, and two coding SNPs in the fibrinogen-like domain alter the substrate binding affinity.[40-42]

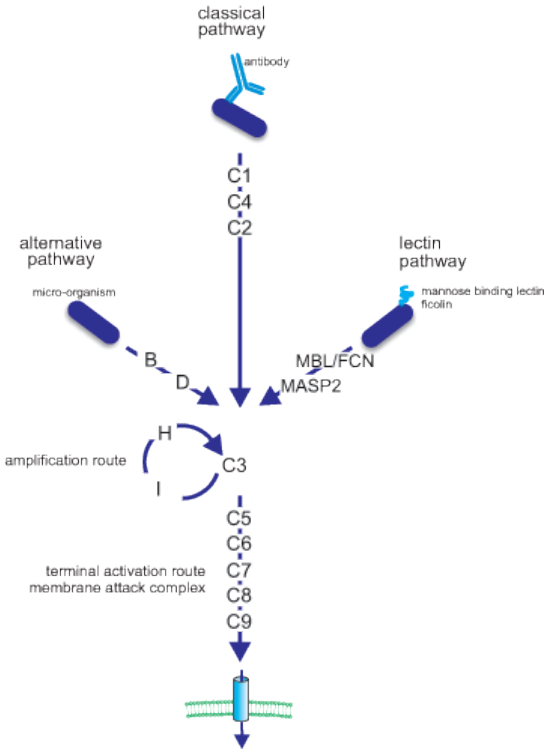


**Figure 3.** Activation of the complement system via the classical, alternative and lectin pathway.

**Inflammatory response**

After recognition of the microbe by PRRs, local phagocytes eliminate the microbe. During a massive invasion of microbes, the recruitment of additional phagocytes to the infected lung might be necessary. Local phagocytes begin to secrete cytokines and chemokines to attract these monocytes and macrophages. Cytokines can be divided into pro- and anti-inflammatory cytokines.[43] The most important pro-inflammatory cytokines are interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α). These cytokines activate phagocytes, synthesise acute phase proteins and are responsible for the systemic inflammatory response (*Figure 4*). Interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1Ra) are the most important anti-inflammatory cytokines and counterbalance the pro-inflammatory response. Interleukin-8 and monocyte chemoattractant protein-1 (MCP-1) are chemokines that mobilise, activate and stimulate degranulation of the polymorphonuclear leukocytes.[44] In experimental settings, the appearance of cytokines depends on the time after the induction of endotoxaemia.[45,46] The specific role of each individual cytokine in the inflammatory response to CAP, as well as their interactions, is still the subject of ongoing research.

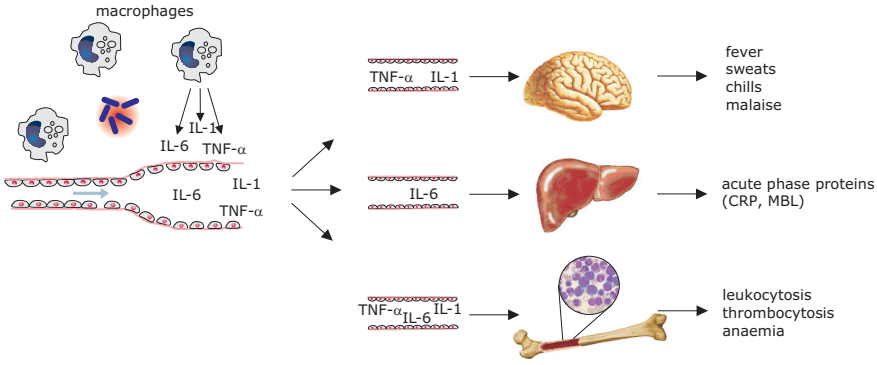


**Figure 4.** The acute phase reaction. The systemic effects of cytokines on brain, liver and bone marrow.

As described above, polymorphisms do occur in genes of the immune system, not only in complement genes but also in the genes for cytokines, cytokine receptors, pathogen recognition receptors, cell signalling pathways etcetera. These polymorphisms can account for a decreased or, in other cases, an overwhelming immune response during an episode of CAP. Due to these genetic variations, some patients have a higher risk for the development of pneumonia or are at greater risk for deterioration.[44,47-50] A better understanding

of the immune response in CAP may, for example, lead to vaccination of patients with an increased risk of pneumonia or therapy to dampen the overwhelming immune response.

**Biomarkers**

The National Institutes of Health's definition of a biomarker is 'a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention'. A more narrow and helpful definition is 'a biochemical feature that can be used to diagnose or assess the progress of disease or the effects of treatment'.[51] To improve the quality of care in patients with CAP, the traditional criteria of infection, which are based on clinical signs, symptoms found by physical examination and laboratory findings, are not always sufficient and/or reliable in the prediction of the severity of CAP.

Biomarkers can be helpful in the management of CAP, including: (1) Confirmation of the diagnosis of CAP. Several non-infectious processes can mimic respiratory symptoms that can be easily confused with bacterial pneumonia; (2) Identification of the potential causative agent. The methods used today to diagnose CAP have not changed substantially since Pasteur and Sternberg first cultured pneumococci from sputum in 1881 and Christian Gram first applied stain to examine sputum specimens in 1886.[51] The overuse of antibiotics for respiratory tract infections is regarded as an important factor in the development of the antibiotic resistance of microorganisms. A better selection of patients with bacterial pneumonia may reduce the unnecessary use of antibiotics; (3) Assessment of severity and risk for adverse outcome. An ideal biomarker can distinguish between patients who can be treated at home and patients with an increased risk for complications who would, therefore, require hospital care; and (4) Identification when a patient has recovered, or conversely the identification of patients with complications. In addition, biomarkers may identify patients who are most likely to benefit from new therapeutic interventions based on immunomodulation, such as corticosteroid treatment. However, all current biomarkers for CAP (*Table 1*) have weaknesses and strengths, and none of the biomarkers can be used on its own. In addition to clinical judgement that is based on clinical, physiological and laboratory features, better biomarkers can help to diagnose or assess the severity of the disease.[52-56]

**Table 1.** Table of available and potential biomarkers in CAP

| | |
|---|---|
| **Biomarkers of inflammation** | Tumor necrosis factor alpha |
| | Lactate |
| | Interleukin-6 |
| | TREM-1 |
| **Biomarkers of coagulation** | Activated partial thromboplastin time |
| | Platelets |
| | Fibrinogen |
| | Disseminated intravascular coagulation scores |
| | Protein C |
| | D-dimer |
| **Biomarkers of infection** | C-reactive protein |
| | Procalcitonin |
| | Blood urea nitrogen |
| | Leukocytes |
| | Endotoxin |
| **Biomarkers of renal function** | Proteinuria |
| | RIFLE criteria |
| **Biomarkers of stress** | Cortisol |
| | Copeptin |

To better identify patients who are at risk for adverse outcomes, one of the possible biomarkers that deserves to be explored is serum ACE activity. ACE activity decreases during pneumonia and returns to normal values during recovery.[57,58] In addition to pneumonia, serum ACE activity is also decreased in adult respiratory distress syndrome (ARDS) and sepsis.[59-63] The clinical significance and biological explanation of the temporary decrease of ACE activity remains unclear, but it may play a role in homeostasis during pneumonia. Another possible biomarker for the prediction of outcomes in CAP might be proteinuria. Proteinuria has been related to both glomerular and tubular dysfunction.[64] The literature has shown that in the general population and in patients with chronic kidney disease (CKD), the presence of proteinuria predicts outcome independent of renal function.[65-69] However, the predictive value of proteinuria during an episode of acute illness, in this case CAP, has not been investigated.

## Adjunctive therapy

Several adjunctive therapies for CAP, including MBL supplementation, TLR-4 modulation, intravenous immunoglobulin administration, and statin therapy, are still under investigation.[70-74] However, which adjunctive therapy will be

best and in which patients is a matter of debate. One of the major targets for adjunctive therapy in CAP is the innate immune response. In most patients, cytokines control and eliminate the primary infection; however, in some patients, the cytokine activation becomes widespread. This widespread activation indicates the need for better control between a sufficient and excessive cytokine response. The extended systemic inflammatory response is presumed to play a role in the organ dysfunction that is characteristic of severe sepsis and septic shock.[75]

Only since the second world war, about 65 years and 2 to 3 generations ago, antibiotics are widely available for the treatment of CAP.[76] Before the age of antibiotics, the only way for a patient to survive pneumonia was through an extensive inflammatory response. Patients with an insufficient inflammatory response were at high risk for death from their pneumonia. However, with the use of antibiotics, it is likely that an overwhelming inflammatory response is not necessary to control the infection and might be harmful.[77]

Adjunctive therapy with the use of corticosteroids can, hypothetically, downregulate the unnecessary excessive cytokine response and accelerate clinical recovery.[78,79] Corticosteroids are the most important physiological inhibitors of inflammation (*Figure 5*). They can switch off genes that encode pro-inflammatory cytokines, and switch on genes that encode anti-inflammatory cytokines.[80] In addition to this direct effect on gene transcription, recent observations have shown that corticosteroids might be effective in patients with severe sepsis due to the relative adrenal insufficiency that is associated with critical-illness and systemic inflammation-induced glucocorticoid receptor resistance.[81] There are different reasons that support a beneficial effect of corticosteroids in not only severe sepsis and septic shock, but also in pneumonia.[82-84] Corticosteroids might be effective in reducing excessive pulmonary inflammation, which reduces lung injury.[85] Furthermore, in some cases of pneumonia, bronchospasm can play a significant role (e.g., in patients with COPD/asthma or viral-induced pneumonia) and can be counteracted by corticosteroids.[86,87] To date, it is unknown whether corticosteroids are effective in CAP and which patients would benefit most from this form of adjunctive therapy.[88-92]

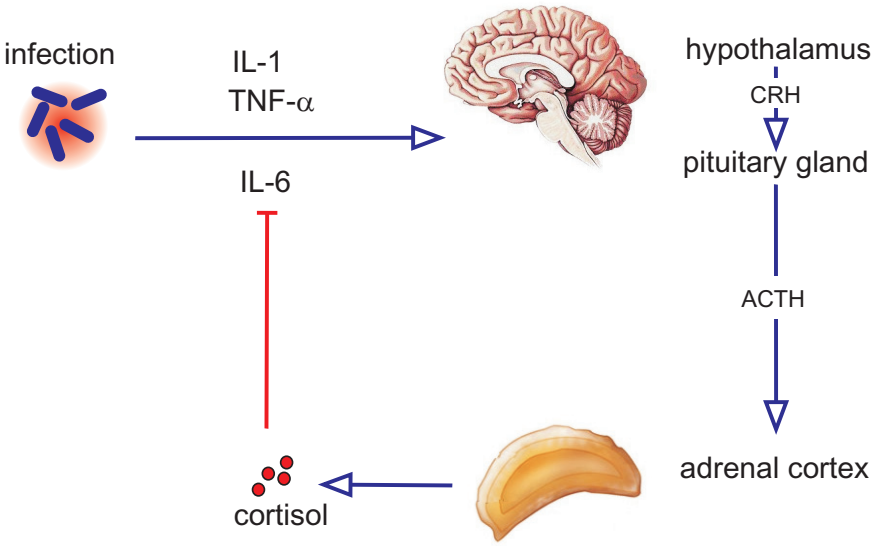


**Figure 5.** Activation of the hypothalamic-pituitary-adrenal gland axis by infection and the interaction with the inflammatory response. IL: Interleukin; TNF-α: Tumour Necrosis Factor-α; CRH: corticotrophin-releasing hormone; ACTH: adrenocorticotrophic hormone.

## Aims and outline of the thesis

The aim of this thesis is to investigate new options in the prevention of community-acquired pneumonia, to improve the understanding of the immune system and to identify biomarkers and adjunctive therapy to improve the quality of care in community-acquired pneumonia.

### Susceptibility to and prevention of CAP

In **chapter 2**, the association between the use of proton pump inhibitors (PPIs) and an increased risk of CAP is examined. Previous results have been conflicting and the causative pathway has never been fully assessed. However, the overgrowth and micro-aspiration of gastrointestinal bacteria are most often proposed as mechanisms for the increased risk of CAP. This association will be further elucidated by including detailed information of microbial aetiology and the clinical characteristics of patients with pneumonia who have recently started PPI treatment in the analyses.

**Immune response in community-acquired pneumonia**

An important part of the innate immune system is the cytokine response. Pro- and anti-inflammatory cytokines are present in the patients intrapulmonary and systemic circulation. In **chapter 3**, the role of these cytokines in CAP in relation to causative microorganisms, the use of antibiotics and clinical course are examined. Furthermore, the role of cytokine polymorphisms (IL-6, IL-8, IL-10, IL-18, TNF-α and RANTES) on cytokine levels in CAP are investigated. And, in a non-randomised setting, the effect of corticosteroids on the cytokine response in patients with CAP are explored.

Next, a different aspect of the innate immune system is analysed; the complement pathway. In **chapter 4**, the role of polymorphisms in MBL and ficolin-2 in patients with recurrent *Staphylococcal aureus* peritonitis, a Gram-positive bacterium are explored. This exploration on the complement system is continued in **chapter 5**. The binding of MBL and ficolin-2 to the pneumococcus is poorly studied and controversial. The aim of chapter 5 is to investigate the binding of MASP-2 to pneumococci and determine whether the eventual binding of MASP-2 results from the binding of MBL and/or ficolin-2. Furthermore, the role of pneumococcal serotype and MBL/ficolin-2 genotype on the activation of the lectin pathway of complement binding are evaluated.

In **chapter 6**, it is evaluated whether a new diagnostic strategy using a longitudinal analysis of pneumococcal antibodies can prove a higher involvement of *S. pneumoniae* in CAP. In contrast to vaccination response studies, few data exist on the immune response during pneumococcal infection. Not only pneumococcal pneumonia patients but also patients infected with another respiratory pathogen or with an unidentified causative agent were included in this study. By analysing antibody responses in these groups, the relative contribution of *S. pneumoniae* to all cases of CAP is aimed to estimate.

**Biomarkers**

In **chapter 7**, the use of serum ACE activity on admission as a biomarker for the course and outcome of CAP is explored. ACE has a wide tissue and cellular distribution and is mostly expressed on the luminal membrane of vascular endothelial cells, particularly the pulmonary endothelium. During pneumonia, inflamed (mostly pulmonary) vascular endothelium produces and releases less enzyme, and therefore, the shedding of ACE from pulmonary vasculature might be impaired. In this chapter, the role of low serum ACE activity on admission as a prognostic marker is evaluated.

Acute kidney injury is a clinical event that is frequently seen as a complication in hospitalised patients with community-acquired pneumonia. Proteinuria has been related to both glomerular and tubular dysfunction and can act as a

biomarker of chronic kidney injury. It is known that the presence of proteinuria predicts outcome, independent of renal function in patients with chronic kidney failure. However, the predictive value of proteinuria during an episode of acute illness, such as CAP, has not been reported.
In **chapter 8**, first, the incidence of proteinuria during CAP is investigated, and then the predictive value of proteinuria on outcome in patients who are admitted with CAP is explored. Furthermore, the predictive value of proteinuria with the other known criteria for acute kidney injury (the RIFLE criteria) in these patients is compared.

**Adjunctive therapy**
In **chapter 9**, it is evaluated which therapeutic targets are already available as adjunctive therapy. The recognition of microbial antigens with complement or Toll-like receptors, improvement of effector mechanisms of the immune response with immunoglobulins and the limiting of the immunopathology that is caused by the cytokine storm with corticosteroids, statins or activated protein C (APC) have been described as such targets. Moreover, certain antibiotics, such as macrolides, can also limit the damage that is caused by an overactive immune system. The evidence for these strategies and the current use of the therapies are described.
In **chapters 10** and **11**, the data of a randomised, placebo-controlled trial on the effect of dexamethasone on the length of stay (chapter 10) and cytokine response (chapter 11) during CAP are described. In chapter 11, it is tried to identify which patients receive the greatest benefit from corticosteroid therapy. A major criticism on corticosteroid therapy in CAP is the fact that only patients with relative adrenal insufficiency are supposed to benefit from this type of therapy. In **chapter 12**, the incidence of adrenal insufficiency in CAP is explored. This chapter will also answer the question whether low levels of cortisol at admission influence outcome and whether cortical level predicts benefit of dexamethasone.

This thesis concludes with a general discussion on how these studies have contributed to a better understanding of the innate immune system during pneumonia. Moreover, the use of corticosteroids in CAP and the patient population that receive the greatest benefit from this therapy are discussed. In addition, the clinical implications and direction of future research are indicated.

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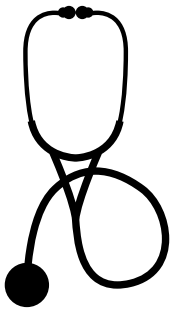
# Part 1

## Susceptibility to and prevention of pneumonia



# Chapter 2

# Microbial evaluation of proton pump inhibitors and the risk of pneumonia

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## Abstract

### Background

Recent initiation of proton pump inhibitor (PPI) treatment may increase the risk of community-acquired pneumonia (CAP), hypothetically by allowing colonisation of the oropharynx by gastrointestinal bacteria. Aim of this study was to assess the causal pathway by considering microbial aetiology of pneumonia and indications for initiation of PPI treatment.

### Methods

This was a population-based case-control study with 430 cases with pneumonia and 1720 matched controls. An elaborate diagnostic protocol was used to identify the causative microorganism of pneumonia. For patients recently starting PPI treatment, indications for treatment were assessed.

### Results

Recent initiation of PPI treatment (<30 days) was associated with an increased risk of CAP (adjusted OR 3.1, 95% CI 1.4 – 7.1). Oropharyngeal bacteria were evenly distributed among current, past and non-users of PPIs (p=0.41). Only in 5 patients (1.2%) with pneumonia (2 current users and 3 non users), gastrointestinal bacteria were identified. Excluding patients who possibly were prescribed PPI treatment for early symptoms of pneumonia (protopathic bias) did not alter the study findings.

### Conclusion

This study reaffirmed that use of PPIs is associated with an increased risk of CAP, especially when treatment is started recently. Neither protopathic bias nor shifts in microbial aetiology seem to explain the association.

## Introduction

Recent evidence has suggested that gastric acid-suppressive medication might increase the risk of community acquired pneumonia (CAP). Results have been conflicting, however, and a meta-analysis failed to draw a definite conclusion due to significant heterogeneity.[1-10] Some researchers are skeptical about the reported association, because causality seemed improbable and results are suspected to be biased.[11-13] Given the widespread use of these medications and the severity of pneumonia, further research remains warranted. So far, most studies used medical record databases to examine the use of proton pump inhibitors (PPIs) in relation to the incidence of CAP. The shortcomings of this approach are inherent to retrospective epidemiologic research on administrative databases. Misclassification of cases might have occurred because clinical information (such as radiographic data) was not always available. Confounding by indication and protopathic bias (when a treatment for the first symptoms of a disease appears to cause the disease) could not be ruled out because most databases did not include information on the indication for PPI treatment. Furthermore, there were no conclusive data on the causative organisms of CAP included in these analyses. Such data would provide more insights into often suggested, but not demonstrated, causal mechanisms, namely overgrowth and microaspiration of gastrointestinal bacteria.

The present study tries to overcome the methodological limitations addressed above by including a well defined cohort of hospitalised CAP patients with elaborate clinical and microbial information and matching them to a population-based control group.

The aim of the present study was to examine the association between use of PPIs and CAP, by including microbial aetiology and clinical characteristics of patients with pneumonia who recently started PPI treatment in the analyses.

## Methods

### Study design

This was a population-based matched case-control study where cases were defined as patients with CAP admitted to the St. Antonius Hospital in Nieuwegein or at the Gelderse Vallei Hospital in Ede, both of which are teaching hospitals (880 and 500 beds, respectively) in the Netherlands. Population-controls were drawn from the PHARMO record linkage system database. The PHARMO institute is an independent scientific research organisation studying drug use and outcomes. Records include detailed information on patient demographics, drug use and hospital admissions, and approximately 3 million community-dwelling inhabitants of 48 geo-demographic areas in the Netherlands are included.[14,15]

**Cases**

Cases were patients with confirmed pneumonia who participated in two clinical trials.[16,17] Consecutive patients were included on the emergency department between October 2004 and August 2006 and between November 2007 and February 2010. Pneumonia was defined as a new infiltrate on a chest radiograph plus at least two of the following criteria: cough; sputum production; temperature >38°C or <35.5°C; auscultatory findings consistent with pneumonia, leukocytosis, or leukopenia (>10 G/L, <4 G/L, or >10% rods in leukocyte differentiation); and C-reactive protein >3 times the upper limit of normal. Patients who were immune compromised (haematological malignancies and immunosuppressive therapy, including the use of >20 mg prednisone equivalent per day for more than 3 days) were excluded. The study was approved by the local Medical Ethics Committee and all patients gave their written informed consent. On the day of hospital admission, the pneumonia severity index (PSI) was calculated.[18] Need for intensive care unit (ICU) admittance and in-hospital mortality were assessed.

**Controls**

Control subjects were obtained from the PHARMO database and individually matched by year of birth, sex and index date to the cases in a 4:1 ratio. The index date was the date of the CAP diagnosis of the corresponding case. Controls with a hospitalisation for CAP during the research period (i.e. in the six months before index date, identified by the International Classification of Diseases, 9th edition (ICD-9-CM) were excluded.

**Pathogen identification**

The diagnostic tools used to identify the causative microorganism of CAP have been described before.[16] In short, at least two sets of separate blood and sputum samples from each patient were cultured. Sputum was analysed by in-house developed polymerase chain reaction for atypical pathogens (*Mycoplasma pneumoniae*, *Legionella pneumophila*, *Coxiella burnetii* and *Chlamydophila pneumoniae* and *psittaci*). Urine was sampled for antigen testing on *Streptococcus pneumoniae* and *Legionella pneumophila* serogroup 1. In addition, serum samples taken on the day of hospital admission and day 10 were analysed in pairs for detection of a fourfold rise of antibodies to respiratory viruses, *Coxiella burnetii*, *Mycoplasma pneumoniae*, and *Chlamydophila psittaci* by complement fixation assay. In addition, antibodies against pneumococcal polysaccharides of 14 different serotypes were measured using the Luminex xMAP® Pneumococcal Immunity Panel.[19] Pharyngeal samples were taken for viral culture. Pathogens were classified in two different ways. First, *Streptococcus*

*pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Haemophilus parainfluenzae* and other streptococci were considered as oropharyngeal bacteria. Second, pathogens considered as gastrointestinal were *Escherichia coli* and *Klebsiella pneumoniae*.

**Exposure definition**

Community pharmacies were approached in order to identify all dispensed prescription drugs for cases issued in the six months before CAP diagnosis. For controls, drug dispensing records were retrieved from the PHARMO database. Exposure definition was identical for cases and controls. PPIs were not over-the-counter available in the Netherlands during the study period. We identified all prescriptions for omeprazole, pantoprazole, lansoprazole, rabeprazole and esomeprazole for both cases and controls. Current use of PPI was defined as a dispensed prescription that lasted beyond 30 days before the index date or started after 30 days before the index date. Past use of PPI was defined as one or more dispensed prescriptions in the 6 months before index date, that did not last beyond 30 days before index date. Non use was defined as no dispensed prescriptions during the 6 months period. These categories were mutually exclusive for each category. A subdivision of the group of current users was made according to the date of the first prescription. Recent initiation was defined as a first prescription <30 days before index date; chronic use was defined as a first prescription ≥30 days before index date. Defined daily doses (DDDs) were calculated based on strength and prescribed dosing regimen of the most recent prescription prior to the index date to express the prescribed daily dose within current users.[20] For all patients who started PPI treatment within 15 days prior to the index date, the indications for starting treatment were assessed through telephonic interview with the patient and or the prescribing physician.

**Potential confounders**

Current use of statins, ACE-inhibitors and angiotensin II receptor antagonists was defined analogous to current PPI use. These drugs have been reported to influence the risk of CAP.[8] Exposure to the following medications was used as a proxy (indicator for disease) for comorbid illness predisposing for CAP and was defined as 2 or more prescriptions in the 6 months before index date. We evaluated use of NSAIDs, antidiabetics (as a proxy for diabetes mellitus (DM)), opiates, antiplatelet therapy, inhalation medication (as a proxy for chronic obstructive pulmonary disease (COPD) or asthma) and digoxine plus diuretics (as a proxy for congestive heart failure (CHF)). Besides this, inhaled corticosteroids and anticholinergics were also evaluated as separate potential confounders.[21,22] Prescriptions for oral corticosteroids during the month before

index date and for antibiotics during the 6 months before index date were also assessed.
The sensitivity of the proxies for DM, COPD or asthma and CHF was checked by studying the consistency of the proxy with the corresponding disease as recorded in the medical charts of cases.

**Statistical analyses**
Conditional logistic regression analysis was performed to obtain crude odds ratios (OR) in matched cases and controls. Results are presented as numbers (percentages), ORs (95% confidence intervals (CI)) and p-value. We considered factors associated with CAP in the univariate analysis and variables previously found to be associated with CAP and PPI use as potential confounders in the multivariate model. We selected potential confounders for the multivariate model stepwise by direct estimation of the degree of confounding produced by each variable (relative change in OR of CAP associated with current use of PPI). We continued including potential confounders to the multivariate model until further addition of confounders modified the OR less than 5%.
A backward logistic regression analysis including age, sex, comorbidities (CHF, COPD or asthma, DM, and renal failure) and PSI score was used to study the outcome of CAP in relation to use of PPIs. The association between PPI use and causative agents of CAP was studied using Chi-square and Fisher's exact tests where appropriate.

## Results

**Characteristics of cases and controls**
The study population comprised 430 CAP cases and 1720 matched controls. Characteristics of cases and controls are shown in *table 1*. The mean age of cases and controls was 62 (SD 18) years and 59% were male. Among cases, 32 patients were admitted to the ICU and 24 patients died during hospital stay. Overall, cases were more likely to use medication than controls.

**Association between use of PPIs and CAP**
*Table 2* lists the crude and adjusted ORs for CAP associated with use of PPIs. In the crude analysis, current use of PPIs was associated with an OR for CAP of 1.8 (95% CI 1.4 – 2.4). In the final multivariate model, oral corticosteroids, inhaled corticosteroids, anticholinergics and NSAIDs were included as confounders. The adjusted OR for CAP associated with current PPI use was 1.6 (95% CI 1.2 – 2.2).
The risk of CAP increased as the starting date of the PPI approached the index date. To ensure that patients identified as new users were not intermittent users, only cases and controls that had not redeemed a prescription for PPIs during

**Table 1.** Characteristics of community-acquired pneumonia cases and controls

| | All patients (n = 2150) | Cases (n = 430) | Controls (n = 1720) | Crude OR (95% CI) |
|---|---|---|---|---|
| **Mean age, years (SD)** | 62 (18) | 62 (18) | 62 (18) | NA |
| **Men** | 1270 (59) | 254 (59) | 1016 (59) | NA |
| **ACE inhibitors** | 343 (16) | 80 (19) | 263 (15) | 1.3 (0.99-1.8) |
| **Angiotensin receptor antagonists** | 210 (9.8) | 32 (7.4) | 178 (10) | 0.69 (0.46-1.0) |
| **Statins** | 478 (22) | 102 (24) | 376 (22) | 1.1 (0.86-1.5) |
| **Antidiabetics** | 255 (12) | 67 (16) | 188 (11) | 1.5 (1.1-2.1) |
| **COPD or asthma drugs*** | 276 (13) | 111 (26) | 165 (9.6) | 3.4 (2.6-4.5) |
| Inhaled corticosteroids* | 234 (11) | 86 (20) | 148 (8.6) | 2.7 (2.0-3.6) |
| Anticholinergics* | 169 (7.9) | 69 (16) | 100 (5.8) | 3.3 (2.4-4.7) |
| No inhalation steroids or anticholinergics | 36 (1.7) | 5 (1.2) | 31 (1.8) | 0.64 (0.25-1.7) |
| **CHF medication** | 50 (2.3) | 19 (4.4) | 31 (1.8) | 2.5 (1.4-4.6) |
| **NSAIDS** | 156 (7.3) | 27(6.3) | 129 (7.5) | 0.82 (0.53-1.3) |
| **Antiplatelet therapy** | 445 (21) | 92 (21) | 353(21) | 1.1 (0.81-1.4) |
| **Antibiotics** | 476 (22) | 104 (24) | 372 (22) | 1.2 (0.97-1.6) |
| **Oral corticosteroids** | 82 (3.8) | 44 (10) | 38 (2.2) | 5.5 (3.4-8.8) |
| **Opiates** | 125 (5.8) | 21 (4.9) | 104 (6.0) | 0.80 (0.49-1.3) |

Date are presented as number (%), unless otherwise stated. NA: not applicable
* Not mutually exclusive categories

the year before index date were included. Patients with a first prescription ≤15 days before index date had an adjusted OR of 3.1 (95% CI 1.1 – 8.8). Patients with a first prescription 16-29 days before index date had an adjusted OR 3.3 (95% CI 0.91 – 11.6). A sensitivity analysis including new users that did receive PPIs during the half-year period of 12 up to 6 months before index date (but did not receive any prescriptions >6 months before index date until <30 days before index date), included 1 new user for cases and 6 new users for controls extra. In this analysis, recent initiation of PPI treatment remained significantly associated with an increased risk for CAP (adjusted OR 2.4 (95% CI 1.1 – 5.0)). As shown in *table 2*, there was a modest dose effect relation for current use of PPIs.

### Clinical details of CAP cases recently starting PPI treatment

*Table 3* provides clinical background information on the pneumonia patients that recently started PPI treatment. Medical history differed markedly between patients, although cardiovascular disease and COPD were common comorbidities. The indications for PPI treatment were diverse as well.

**Table 2.** Odds Ratios (ORs) for community-acquired pneumonia associated with use of proton pump inhibitors (PPIs).

| | | | | Odds Ratios | | | |
|---|---|---|---|---|---|---|---|
| | **All** | **Cases** | **Controls** | **Crude OR** | | **Adjusted OR*** | |
| | **(n = 2150)** | **(n = 430)** | **(n = 1720)** | **(95% CI)** | **p-value** | **(95% CI)** | **p-value** |
| **Non user** | 1690 (79) | 307 (71) | 1383 (80) | reference | | reference | |
| **Past user** | 90 (4.2) | 20 (4.7) | 70 (4.1) | 1.3 (0.78 – 2.2) | 0.31 | 1.2 (0.72 – 2.1) | 0.46 |
| **Current user** | 370 (17) | 103 (24) | 267 (16) | 1.8 (1.4 – 2.4) | <0.01 | 1.6 (1.2 – 2.2) | <0.01 |
| **Start of PPI treatment †** | | | | | | | |
| Recent (<30 days) | 28 (7.6) | 12 (12) | 16 (6.0) | 3.4 (1.6 – 7.3) | <0.01 | 3.1 (1.4 – 7.1) | <0.01 |
| 0 – 15 days | 16 (4.3) | 7 (6.8) | 9 (3.4) | 3.5 (1.3 – 9.6) | 0.01 | 3.1 (1.1 – 8.8) | 0.04 |
| 16 – 29 days | 12 (3.2) | 5 (4.9) | 7 (2.6) | 3.3 (1.1 – 10.4) | 0.04 | 3.3 (0.91 – 11.6) | 0.07 |
| Chronic (≥30 days) | 342 (92) | 91 (88) | 251 (94) | 1.7 (1.3 – 2.3) | <0.01 | 1.5 (1.1 – 2.1) | <0.01 |
| **DDD‡** | | | | | | | |
| <1.5 | 292 (79) | 80 (78) | 212 (79) | 1.8 (1.3 – 2.4) | <0.01 | 1.6 (1.2 – 2.2) | <0.01 |
| ≥1.5 | 78 (21) | 23 (22) | 55 (3.2) | 2.0 (1.2 – 3.3) | 0.01 | 1.7 (1.0 – 3.0) | 0.05 |

Data are presented as number (%)
Adjusted OR: adjusted for use of inhalation corticosteroids, anticholinergics, NSAIDs and oral corticosteroids
† Days from first prescription until index date
‡ DDD Defined Daily Doses, analysis within current users

**Table 3.** Case summaries of pneumonia patients who recently started proton pump inhibitor (PPI) treatment

| Case no | Age | Sex | Medical history | Indication for PPI use | PPI | DDD | Days to CAP | Causative organism | PSI score | Admission to ICU |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 60 | f | Resection middle lobe because of recurrent pneumonia caused by bronchiectasis (5 years before CAP), Primary biliary cirrhosis | Gastric protection during NSAID use for artrosis in hands | O | 1 | 2 | *H. influenzae* | 60 | No |
| 2 | 72 | m | Hypertension, COPD (GOLD 1) | Gastric protection during NSAID use for lower back pain | O | 1 | 10 | *E. coli* | 102 | No |
| 3 | 80 | m | Aorta valve replacement after stenosis (6 years before CAP). No further cardial pathology | Gastric protection during NSAID use for lower back pain | O | 2 | 13 | Parainfluenzae virus | 90 | No |
| 4 | 18 | m | - | Gastric discomfort and air regurgitation | O | 1 | 20 | *S. pneumoniae* | 58 | No |
| 5 | 34 | f | Deep venous thrombosis (1 month before CAP) | Gastric protection during NSAID use for painful leg. Previous use of NSAID caused discomfort | O | 1 | 25 | unidentified | 34 | Yes |
| 6 | 65 | f | COPD, transient ischemic attack, breast carcinoma (curative treatment 3 years before CAP) | Gastric protection during use of NSAID, prednisone, aspirin and dipyridamole | P | 1 | 8 | *H. influenzae* | 105 | Yes |
| 7 | 70 | m | Pneumonia, hypertension, hypercholesterolemia | Xyphoid pain radiating to lungs, general practitioner assumed GERD, but it might have been CAP. | O | 1 | 1 | unidentified | 60 | No |
| 8 | 32 | m | - | Gastric protection during NSAID use for painful shoulder | O | 1 | 8 | *S. milleri* | 57 | No |
| 9 | 41 | f | Uterus myomatosis | Stomach ache. Patient reports to always experience stomach ache before fever | P | 1 | 1 | *L. pneumophila* | 51 | No |
| 10 | 83 | f | Aorta valve stenosis, mitralis valve insufficiency, hypertension, COPD, CHF | Stomach ache during *Helicobacter pylori* infection | P | 1 | 29 | *S. pneumoniae* | 83 | No |
| 11 | 60 | f | Alcohol abuse, DM II | Therapy for bleeding duodenal ulcer | O/P * | 1 | 16 | unidentified | 80 | Yes |
| 12 | 62 | m | DM II, hypertension, COPD with emphysema, CHF, pulmonary hypertension | Therapy for peptic ulcer during NSAID use | R | 1 | 22 | *S. pneumoniae* | 122 | Yes |

Abbreviations: CAP: community-acquired pneumonia; COPD: chronic obstructive pulmonary disease; CHF: congestive heart failure; DDD: defined daily dose; DM II: diabetes mellitus type II; GERD: gastroesophageal reflux disease; ICU: intensive care unit; O: omeprazole, P: pantoprazole; PPI: proton pump inhibitor; PSI: pneumonia severity index; R: rabeprazole

*This patient received an additional prescription for pantoprazole 40 mg daily 7 days after the omeprazole prescription was issued, 9 days before CAP diagnosis. Based on the prescribed daily dose, the prescription for omeprazole could have lasted until 1 day before CAP diagnosis.

Patient number 7 received a PPI for xyphoid pain, which might have been CAP- and not reflux related. Patient number 9 also experienced possible symptoms of CAP. Half of the patients received a PPI as prophylaxis for gastrointestinal bleeding and ulcera due to NSAIDs. There was no reason to suspect that their pain complaints (e.g. lower back pain) were early symptoms for CAP. The indications for the remaining cases were (bleeding) ulcera, *Helicobacter pylori* infection and dyspepsia.
In order to assess whether protopathic bias could explain the demonstrated increase in risk associated with recent initiation of PPI treatment, we conducted a sensitivity analysis by considering case number 7 and 9 non-exposed. The risk for CAP remained significantly elevated for recent initiation of PPI treatment (adjusted OR 2.5, 95% CI 1.0 – 5.8). If case numbers 1, 7 and 9 were excluded (because prescriptions were issued 2 or less days before CAP diagnosis, which could be too short to produce an acid suppressive effect and subsequent change in commensal flora) the adjusted OR yielded 2.1 (95% CI 0.85 – 5.1).

**Clinical outcomes**
Four of the 12 patients (33%) who recently started PPI treatment (<30 days before admission) were admitted to the ICU, whereas only 7% and 11% of non and current users, respectively, were admitted to the ICU. After adjusting for comorbidities, age, sex and PSI score, recent initiation of PPI treatment was independently associated with ICU admission ($p<0.01$). Chronic and past use were not associated with ICU admission ($p=0.89$ and $p=0.99$ respectively). None of the patients who recently started PPI treatment died during hospital stay.

**Causative pathogens**
Among CAP patients, *Streptococcus pneumoniae* was identified in 30% of the cases. In 36% of cases, a causative organism could not be identified. *Table 4* shows the microbial aetiology for current, past and non users of PPIs.
In the 430 CAP cases, five were caused by defined gastrointestinal bacteria. Three of these (1%) were not receiving PPI treatment, two (2%) were current users of PPIs. Defined oropharyngeal bacteria were identified in 41% of current users, 25% of past users and in 39% of non users (versus non-oropharyngeal and unidentified pathogens, $p=0.41$). The frequency of oropharyngeal pathogens did not differ between patients recently starting PPI treatment and non users (versus non-oropharyngeal and unidentified pathogens, $p=1.00$).

**Table 4.** Causal pathogens of community-acquired pneumonia in non, past and current users of proton pump inhibitors.

| | All (n =430) | Non users (n=307) | Current users (n=103) | Past users (n=20) |
|---|---|---|---|---|
| ***Streptococcus pneumoniae*** | 130 (30) | 97 (32) | 28 (27) | 5 (25) |
| **Atypical** | 69 (16) | 54 (18) | 7 (6.8) | 8 (40) |
| **Viral** | 25 (5.8) | 20 (6.5) | 5 (4.9) | 0 (0) |
| **Gram negative** | 37 (8.6) | 24 (7.8) | 13 (13) | 0 (0) |
| **Other** | 15 (3.5) | 9 (2.9) | 6 (5.8) | 0 (0) |
| **Unidentified** | 154 (36) | 103 (34) | 44 (43) | 7 (35) |
| **Oropharyngeal bacteria identified*** | 166 (39) | 119 (39) | 42 (41) | 5 (25) |
| **Gastrointestinal bacteria identified†** | 5 (1.2) | 3 (1.0) | 2 (1.9) | 0 (0) |

Data are presented as number (%)
*Bacteria considered as oropharyngeal were *S. pneumoniae*, *H. influenzae*, *S. aureus*, *H. parainfluenzae* and other streptococci. Use of PPIs was not associated with causation of CAP by oropharyngeal bacteria (tested to non oropharyngeal and unidentified pathogens, p-value=0.41).
† Bacteria considered as gastrointestinal were *E. coli* and *K. pneumoniae*.

## Performance of proxies in cases

The results of the comparison of our proxies with recorded medical diagnoses are shown in *figure 1*.

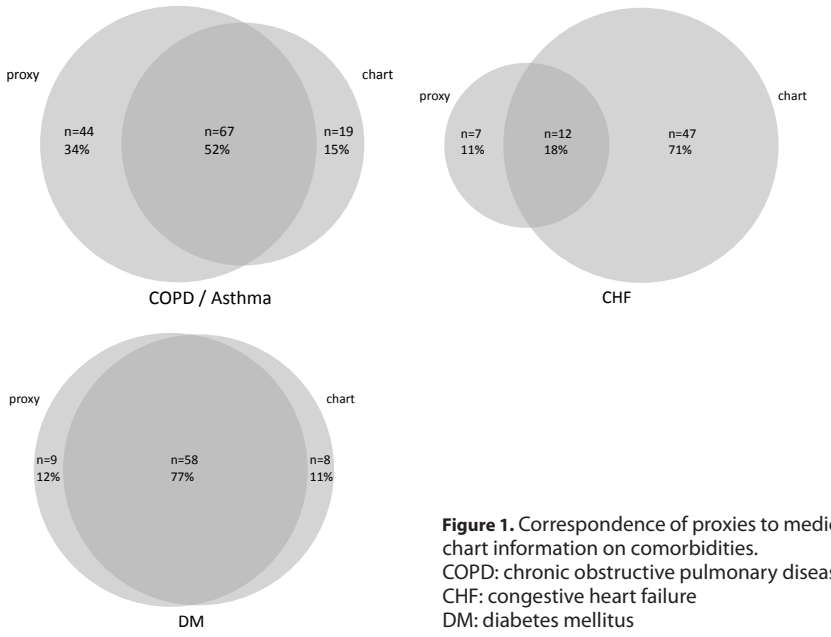


**Figure 1.** Correspondence of proxies to medical chart information on comorbidities.
COPD: chronic obstructive pulmonary disease
CHF: congestive heart failure
DM: diabetes mellitus

## Discussion

In this study, the risk of CAP was increased in patients currently using a PPI. We confirmed that the risk was highest shortly after initiation of PPI treatment. Because of this seemingly contradictory timing effect, we further examined the CAP patients who recently started PPI treatment. It became clear that protopathic bias is not the sole explanation for the observed risk. Study of the causative microorganism of CAP did not show an increase in the frequency of either oropharyngeal or gastrointestinal bacteria in patients using PPIs.

Laheij *et al*.[7] were the first to report a positive association between current use of PPIs and risk of CAP. Most, but not all subsequent studies confirmed this association and also described a gradual increase in effect size when treatment was started closer to the index date. As maximum acid suppression is reached after 7 days of PPI treatment, this pattern of association is difficult to account for. Protopathic bias has been put forward as a possible explanation: patients presenting with CAP-related cough might be misdiagnosed as having gastroesophageal reflux disease (GERD) or patients presenting with CAP-related pain might be prescribed an NSAID with a PPI for prophylaxis. Our study is the first to provide detailed information on patients who recently started PPI treatment, for whom the supposed association is most controversial. Only two of these patients received a prescription intended for complaints that might have been linked to early pneumonia, and a sensitivity analysis excluding these cases showed that the observed association remained increased.

Previous reports have suggested that backflow and overgrowth of gastrointestinal bacteria during PPI treatment may result in colonisation of the oral space and predispose to pneumonia. Although such a mechanism has been demonstrated in mechanically ventilated patients, it remains speculative in CAP. [23-25] The current study was the first to include elaborate microbial data, acquired using an extensive diagnostic protocol to identify the causative agent of CAP. As in only two (2%) current PPI users CAP was caused by gastrointestinal bacteria, overgrowth and aspiration of gastrointestinal flora seems not the most prominent cause of pneumonia during acid-suppressive treatment. Our alternative hypothesis was that overgrowth of oropharyngeal bacteria during PPI treatment predisposes patients for developing CAP. Plausibly, clearance of oropharyngeal bacteria is reduced when the pH of aspirated gastric contents is increased and possibly of the oropharyngeal fluid, as the proton pump is also assumed to be present in the larynx.[26-28] However, the frequency of CAP caused by bacteria that typically colonise the oropharynx was not increased in patients using a PPI. Five cases of pneumonia (42%) from the 12 patients recently starting PPI treatment were caused by oropharyngeal bacteria. Thus, also in the group in which the risk of CAP is the highest, overgrowth of either gastrointestinal or

oropharyngeal bacteria does not seem to explain the association between use of PPIs and risk of CAP.

Given our findings that revoke microbial or non causal pathways as underlying mechanisms of the association; future research should directed towards other PPI properties or other types of bias. One possible explanation could be the immunomodulatory effects of PPIs. Omeprazole and lansoprazole have been shown to inhibit the expression of adhesion molecules on neutrophils, indicating that PPIs may diminish adequate transmigration of leukocytes to inflammatory sites.[29,30] In a small study of ten healthy volunteers, a single oral dose of omeprazole 40mg decreased reactive oxygen production and neutrophil bactericidal activity.[31] Experimental evidence suggests that omeprazole elevates intralysosomal pH, through inhibition of the neutrophil proton pump, thereby reducing the production of toxic oxidants.[32,33] In the present study we were unable to explore this possible causal pathway.

The major weaknesses of our study are inherent to its observational design. Residual confounding might be present as we did not have information on the indications for the PPI treatment of all patients, nor on medical diagnoses and lifestyle of controls. Instead we used proxies to identify comorbidities (COPD or asthma, CHF and DM). As shown in *figure 1*, the proxies for COPD or asthma and DM are very reliable. Remarkably, COPD or asthma were present according to proxy but not recorded in the chart in 34% of all cases with COPD or asthma. As only 15% of cases had COPD or asthma that was not identified by our proxy, it seems that the proxy might even perform better than medical record scoring, as it is unlikely that patients would receive and fill two or more prescriptions for airway medication if disease is not present. In the Netherlands, it is possible that a general practioner treats a patient with mild COPD or asthma. The proxy used for CHF is less consistent with medical record scoring. However, the number of CHF patients identified by physicians is low, therefore the impact of the disease as a possible confounder would remain moderate, also with better performance of proxies.

Regarding lifestyle, as no such information was available of the controls, to evaluate the possibility of confounding we searched for associations between both smoking and alcohol abuse and use of PPI treatment within the pneumonia patients. This analysis showed that cases who smoked were less likely to use PPIs than non-smoking patients (11% vs. 28%) and that there was no difference for excessive alcohol use or none (22% vs. 23%). Considering that these habits are risk factors for pneumonia, this could indicate an underestimation of the true association between PPI use and pneumonia in our study.

Another limitation could be the origin of the controls. Instead of hospital controls we selected population controls. We feel confident that population controls

represent better the population from which our cases originated. The PHARMO database hold a very representative sample of the Dutch population and a prior study showed that the patients admitted with pneumonia to the St. Antonius Hospital Nieuwegein resemble patients studied in PHARMO very much.[34] The prevalence of PPI use is comparable for all parts of the Netherlands.[35] Finally, an issue that can only be addressed in a randomised controlled trial is that of poor adherence. Prescriptions for PPIs do not directly reflect exposure to PPIs and patients who are being prescribed a PPI for prophylaxis of gastrointestinal ulcera, will adhere less to therapy than patients with active ulcera or dyspepsia. This might be the reason that the risk seems to fade out as PPI therapy turns chronic, because continue use will often reflect prophylactic therapy, whereas short term use will mainly be indicated in active ulcera.

In conclusion, recent initiation of PPI treatment is associated with an almost threefold increase in the risk of CAP. Study of the patients recently prescribed PPI treatment, showed that the association is not likely to be attributable to protopathic bias. Neither gastrointestinal nor oropharyngeal bacteria were more present in patients using a PPI compared to patients not using a PPI. Given these findings, further study on the causal pathway of the increased risk for pneumonia during PPI use should be directed towards other PPI properties.

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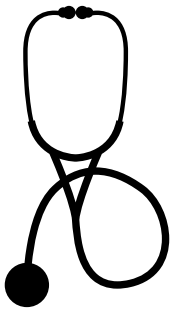
# Part 2

## Immune response in community-acquired pneumonia



# Chapter 3

# Systemic cytokine response in patients with community-acquired pneumonia.

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## Abstract

### Background

The role of individual cytokines and polymorphisms in pneumonia has been described, but the relation between different cytokines and polymorphisms in relation to causative microorganisms, antibiotics, corticosteroids and clinical course has not. This study questions the relationship between cytokines, polymorphisms and clinical characteristics of pneumonia.

### Methods

Patients diagnosed with pneumonia were included in the study. Serum cytokine levels were measured during hospital stay, genotyping was performed, causative microorganisms were identified and patients were monitored throughout the hospital stay.

### Results

In 201 patients with pneumonia, interleukin (IL)-1 receptor antagonist (IL-1Ra), IL-6, IL-8 and IL-10 acted as acute phase proteins. After admission, the levels of these cytokines decreased rapidly. Single nucleotide polymorphisms did not influence cytokine production and were not associated with clinical outcome. Cytokine serum levels were significantly higher in patients with pneumococcal pneumonia. The decrease in levels of cytokines was independently influenced by the start of corticosteroid therapy.

### Conclusion

IL-1Ra, IL-6, IL-8 and IL-10 are acute phase proteins, independent of genotype. Their levels are influenced by the nature of the causative microorganism and the start of corticosteroid therapy.

## Introduction

Community-acquired pneumonia (CAP) is the leading cause of community-acquired infection needing hospitalisation, and has an overall mortality of 8.7%.[1] The human immune response to CAP varies between the different causative microorganisms and severities of disease. An important early component of the host response is the release of cytokines, produced by inflammatory cells.[2,3] In experimental settings, the appearance of cytokines depends on the time after induction of endotoxaemia. The role of each specific cytokine in the inflammatory response to CAP is still a subject of ongoing research. In general, tumour necrosis factor (TNF)-α and interleukin (IL)-6 are considered essential pro-inflammatory proteins, and IL-10 is considered the most important cytokine with anti-inflammatory properties.[2,4-7] These three cytokines are reported to play an important role in the susceptibility to experimental pneumonia caused by different pathogens.[8-10] Chemokines are locally produced cytokines and their main function is to attract and activate macrophages and monocytes, thereby involving them in the primary clearance of various bacteria.[11,12]
In sepsis, systemic cytokine levels are associated with the severity of disease.[13,14] In bacterial pneumonia the cytokine response is mostly confined to the affected lung[15-18], but systemic levels of cytokines are also raised.[15,16,18-20] The severity of pneumonia is both reflected and predicted by higher levels of cytokines in blood.[18-22] Polymorphisms within the genes coding for cytokine expression are also associated with severity of disease.[13,23-29]
The role of individual cytokines and cytokine polymorphisms is well known. Herein, we question the relationship between cytokines, polymorphisms and clinical characteristics of CAP. Therefore, we describe the systemic cytokine response of different cytokines and cytokine polymorphisms in CAP in relation to causative microorganisms, the use of antibiotics and corticosteroids and clinical course. Furthermore, the role of cytokine polymorphisms on cytokine levels in CAP is reported.

## Methods

### Patients and controls

From October 2004 until August 2006, patients with confirmed pneumonia admitted to the emergency department (ED) of the St. Antonius Hospital (Nieuwegein, the Netherlands) were included in this study. Pneumonia was defined as an new infiltrate on chest radiograph in combination with at least two of the following criteria: cough, sputum production, temperature >38°C or <35°C, auscultatory findings consistent with pneumonia, C-reactive protein (CRP) >15 mg/L, or leukocytosis or leukopenia (white blood count

>10x10$^9$/L, <4x10$^9$/L, respectively, or >10% rods in leukocyte differentiation).[30] Patients with defined immunodeficiencies (a known congenital or acquired immunodeficiency, chemotherapy within the last 6 weeks, corticosteroids use in the last 6 weeks (prednisone equivalent >20 mg/daily for more than 3 days), immunosuppressive medication in the last 6 weeks), haematological malignancies or recent hospitalisation (<30 days) were excluded. At inclusion, clinical and laboratory parameters were recorded, and the Pneumonia Severity Index (PSI) was calculated.[31] Blood was collected and serum samples stored to determine cytokine profiles by multiplex immunoassays. Mortality, intensive care unit (ICU) admission, length of hospital stay, and the causative microorganism were assessed. Written informed consent was obtained from all patients. The study was approved by the institutional medical ethical committee (Verenigde Commissie Mensgebonden Onderzoek, St. Antonius Ziekenhuis, Nieuwegein, the Netherlands.

**Pathogen identification**

The diagnostic tools used to identify the causative microorganism of CAP have been described previously.[32] In short, at least two sets of separate blood and sputum samples from each patient were cultured. Urine antigen tests were performed for the detection of *Legionella pneumophila* serogroup 1 and *Streptococcus pneumoniae*. In-house developed PCR were performed on the sputum to detect *L. pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae*/*psittaci*. Serological testing was performed for the presence of antibodies to *M. pneumoniae*, *Coxiella burnetii*, or respiratory viruses (influenza and parainfluenza viruses, adenovirus, respiratory syncytial virus). Pharyngeal samples were taken for viral culture (influenza viruses).

**Determination of systemic cytokine level**

Systemic circulating concentrations of IL-1 receptor antagonist (IL-1Ra), IL-5, IL-6, IL-8, IL-10, IL-12, interferon (IFN)-γ, macrophage inflammatory protein (MIP) and monocyte chemoattractant protein-1(MCP-1) were measured on day of presentation and subsequent samples were drawn at 08:00 h on days 2, 3, 5, and 10 and at a control visit at least 30 days after admission (convalescent phase). For technical details of analysis of cytokine levels see appendix A. To analyse whether these cytokines and chemokines act as acute phase proteins, we analysed the difference in cytokine levels between the acute and convalescent phases. An acute phase response was defined as a decrease or increase in the cytokine or chemokine level by at least 25% in the acute phase compared with the convalescent phase.[33]

**DNA isolation and genotyping**

A 200-µl whole blood sample was used to extract DNA with the MagNA Pure LC DNA isolation kit I (Roche Diagnostics, Mannheim, Germany) for genotyping. Genotyping was performed on an 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the TaqMan® technique with customised primers and probes designed by Applied Biosystems using the manufacturer's instructions. Technical details of genotyping, including primer sequences are given in appendix B. Individual haplotypes were inferred using PHASEv2 software and analysed to determine when frequencies exceeded a 5% threshold.[34] Distribution of genotypes was compared with the distribution of genotypes in 313 healthy unrelated Dutch volunteers with the same geographical background as the patients.

**Statistical analyses**

All statistical analyses were performed using statistics software (SPSS, Inc., Chicago, IL, USA) and a two-tailed p-value of $<0.05$ was considered significant. Differences in continuous variables were tested by the t-test (tested within patients, paired; tested between patients, unpaired) or the Mann-Whitney U- test, as appropriate. Genotype frequencies of CAP patients were tested for conformity to Hardy-Weinberg equilibrium using the Chi-square test between the observed and expected numbers. Correlations were tested using the Spearman's test. We conducted a linear regression analysis including age, sex, PSI score, chronic obstructive pulmonary disease (COPD), causative microorganism (pneumococcal versus nonpneumococcal), antibiotic therapy prior to hospitalisation and the use of corticosteroids and IL-6 concentration on day 1 to predict IL-6 concentration on day 3 (dependent variable). For this linear regression the cytokines were transformed into a natural log scale, because the cytokines were not normally distributed. In the linear regression analysis we chose to look at the decrease in IL-6 from day 1 to day 3 because all patients using corticosteroids were on corticosteroids for least 24 h on day 3 and because cytokine concentrations decreased the most in the first few days. To correct for the height of IL-6 on day 1, this is a independent variable in the linear regression. A similar multivariate analysis was performed to search for an effect of polymorphisms on cytokine levels, including age, comorbidities (COPD and heart failure) and PSI score.

## Results

In 201 patients, a new infiltrate on chest radiograph was confirmed by an experienced radiologist. We obtained blood for the assessment of cytokine concentrations from 171 of the 201 enrolled patients. DNA was isolated from

200 of the 201 patients with CAP. The baseline characteristics of these patients are shown in *table 1*. In 64% of the patients the causative microorganism for the CAP could be identified.

**Table 1.** General characteristics of 201 hospitalised patients with community-acquired pneumonia

| | |
|---|---|
| **Age, years** | 63.7 ±17 |
| **Sex, male** | 124 (62) |
| **Comorbidity** | |
| Diabetes Mellitus | 35 (17) |
| CVA | 17 (9) |
| COPD | 63 (31) |
| Hypertension | 38 (19) |
| **Nursing home residents** | 3 (1) |
| **Pneumonia Severity Index**: | |
| Low I | 30 (15) |
| Low II | 34 (17) |
| Low III | 53 (26) |
| Moderate IV | 56 (28) |
| High V | 28 (14) |
| **ICU admission** | 21 (10) |
| **Mortality** | 10 (5) |
| **Microbiological species:** | |
| *Streptococcus pneumoniae* | 60 (30) |
| *Haemophilus influenzae* | 14 (7) |
| *Legionella pneumophila* | 9 (4) |
| *Mycoplasma pneumoniae* | 9 (4) |
| Other | 36 (18) |
| Unknown | 73 (36) |

Data presented as mean (SD) or number (%).
CVA: cerebrovascular accident (stroke); COPD: chronic obstructive pulmonary disease; ICU: intensive care unit

Cytokine and chemokine serum levels at admission and during hospital stay

The levels of the 9 cytokines and chemokines (and CRP) were determined at admission and on consecutive days. *Figure 1* shows that the levels of IL-1Ra, IL-6, IL-8, IL-10, and MCP were highest at admission. An acute phase response was observed for IL-6 (+1695% as compared to day 30 levels), CRP (+1088%), IL-1Ra (+450%), IL-10 (+332%), and IL-8 (+96%) (*Figure 2*). For MCP (+22%)

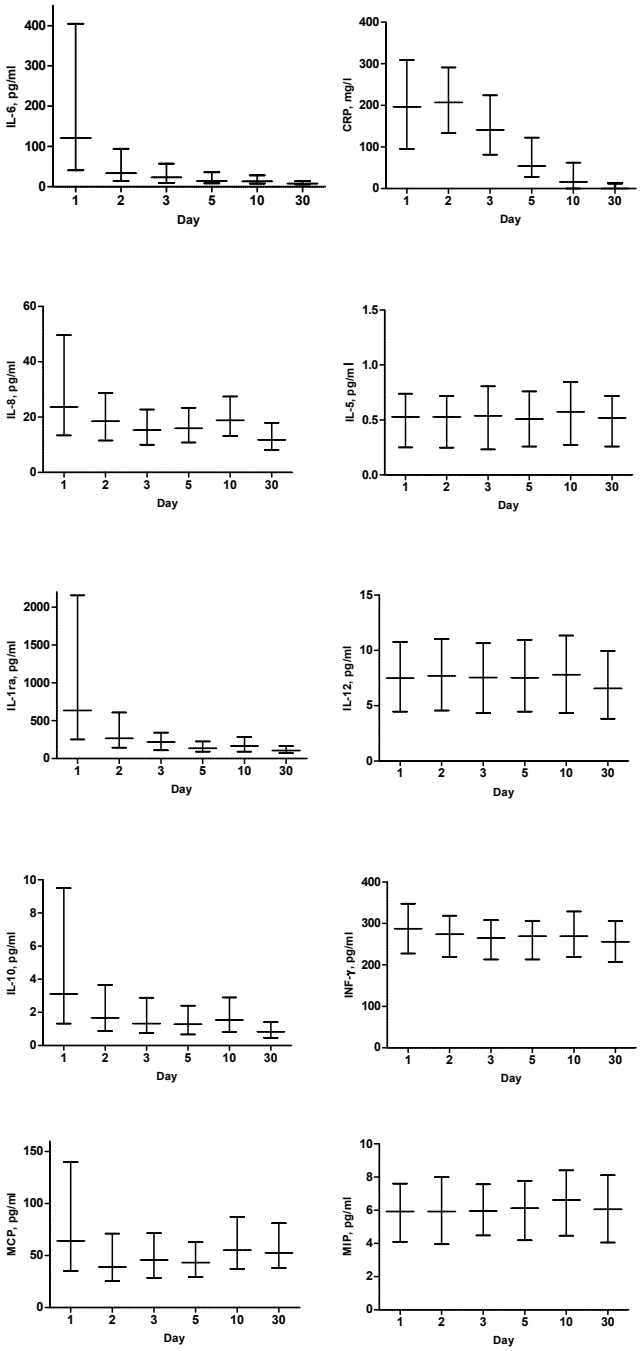



**Figure 1.** Median plasma cytokine concentrations with the interquartile range on subsequent days.
a) Interleukin (IL)-6, b) C-reactive protein (CRP), c) IL-8, d) IL-5, e) IL-1 receptor antagonist (IL -1Ra), f) IL-12, g) IL-10, h) interferon (IFN)-γ, i) monocyte chemoattractant protein-1 (MCP-1) and j) macrophage inflammatory protein (MIP).

there was a trend towards an acute phase response. In contrast to acute phase cytokines, the levels of CRP remained at a high level at day 2 (199 mg/L and 201 mg/L at days 1 and 2, respectively) and decreased thereafter. Levels of the other measured cytokines and chemokines did not show an acute phase response after admission and during the hospital stay: IL-12 (+6%), INF-γ (+6%), IL-5 (0%), and MIP (-4%). We analysed if there were differences in cytokine response between survivors and a combined group of patients (non-survivors and patients who needed admission to the ICU); however, due to lack of power (only 10 patients deceased and 21 patients were admitted to the ICU) we were unable to show statistically significant differences. We did not find a relation between cytokine levels and/or kinetics and length of hospital stay.

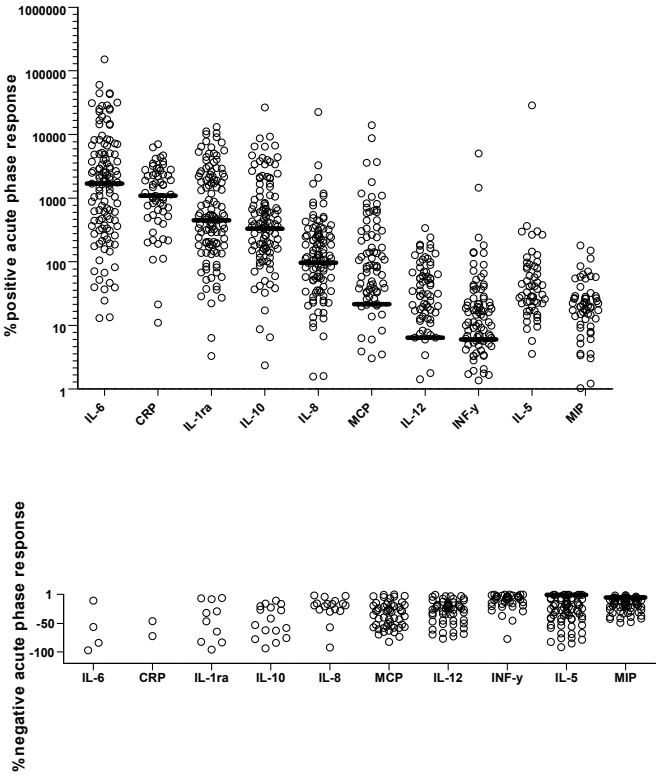



**Figure 2.** Dynamics of the measured cytokines in CAP patients. The relative change (%) of the cytokines in the acute phase of disease compared to the convalescent phase is plotted. The cut-off for positive (+25%) and negative (-25%) acute phase responses are plotted (drawn line). Data points of individual patients are shown as open circles; horizontal bars indicate median values. a) Positive acute phase responses are indicated in the upper panel on a logarithmic scale and b) negative acute phase responses are indicated in the lower panel on a linear scale. IL: interleukin; CRP: C-reactive protein; IL-1Ra: IL-1 receptor antagonist; MCP: monocyte chemoattractant protein; IFN: interferon; MIP: macrophage inflammatory protein.

**Polymorphisms**

An analysis of 200 patients with CAP for single nucleotide polymorphisms (SNPs) in the IL-6, IL-8, IL-10, IL-18, TNF-α and RANTES genes was performed. The group did not deviate significantly from the Hardy-Weinberg equilibrium ($p>0.05$). We did not find differences in distribution of the tested SNPs between patients and a control group of Dutch healthy unrelated volunteers (*Table 2*). We did not find differences within patients for clinical course, defined as 'need of ICU admission', 'in-hospital mortality' and 'length of hospital stay' between the different tested genotypes. We found no relationship between cytokine levels and polymorphisms, even in multivariate analysis correcting for age, comorbidities and PSI score. The data do not indicate significantly higher cytokine production associated with a specific SNP of IL-6, IL-8 or IL-10 gene. The three SNPs in the IL-10 gene produced four haplotypes (-1082, -592, +3367: ACG, AAG, GCG and GCA). Each of those IL-10 haplotypes showed similar magnitudes and kinetics of IL-10 production.

**Causative microorganisms**

We compared the serum cytokine profiles of patients with pneumococcal pneumonia to patients with another identified microorganism. After adjustments for age, PSI and the use of corticosteroids, IL-6 levels on admission were significantly higher in patients with pneumococcal pneumonia compared to nonpneumococcal pneumonia ($p<0.01$) (*Figure 3*). Similar results were found for the anti-inflammatory cytokine IL-1Ra on admission ($p<0.01$). During the subsequent days, IL-6 and IL-1Ra concentrations fell rapidly to the same (elevated) levels of patients with nonpneumococcal pneumonia. The other cytokine levels did not differentiate between patients with pneumococcal and nonpneumococcal pneumonia at admission or at the following sample days. Results were similar when we compared pneumococcal pneumonia versus atypical pneumonia (*L. pneumophila*, *M. pneumoniae*, and *C. pneumoniae*/*psittaci*).

**Table 2.** Distribution of polymorphisms in patients with community-acquired pneumonia (CAP) and healthy unrelated volunteers.

| SNP (RS-number) | Genotype | CAP[#] | Controls[¶] |
|---|---|---|---|
| IL-6 -174 G/C (RS1800795)[1] | GG | 83 (42) | 113 (36) |
| | GC | 92 (46) | 150 (48) |
| | CC | 25 (13) | 48 (15) |
| IL-6 -572 G/C (RS1800796)[1] | GG | 184 (92) | 283 (91) |
| | GC | 15 (8) | 28 (9) |
| | CC | 1 (1) | 0 |
| IL-8 -251 T/A (RS4073)[2] | TT | 46 (23) | 62 (20) |
| | TA | 94 (47) | 153 (49) |
| | AA | 59 (29) | 98 (31) |
| IL-10 -592 C/A (RS1800872) | CC | 115 (57) | 173(55) |
| | CA | 70 (35) | 126 (40) |
| | AA | 15 (8) | 14 (5) |
| IL-10 -1082 G/A (RS1800896) | GG | 54 (27) | 74 (24) |
| | GA | 90 (45) | 170 (54) |
| | AA | 56 (28) | 69 (22) |
| IL-10 +3367 G/A (RS3024495) | GG | 134 (67) | 225 (72) |
| | GA | 63 (31) | 77 (25) |
| | AA | 3 (2) | 11 (3) |
| IL-18 -137 G/C (RS187238) | GG | 109 (55) | 168 (54) |
| | GC | 79 (40) | 121 (39) |
| | CC | 12 (6) | 24 (8) |
| TNF-α -238 G/A (RS361525) | GG | 187 (93) | 286 (91) |
| | GA | 12 (6) | 29 (8) |
| | AA | 1 (1) | 1 (0) |
| RANTES 1.1 G/A (RS2280789) | GG | 3 (2) | 10 (3) |
| | GA | 51 (25) | 76 (24) |
| | AA | 146 (73) | 227 (73) |

Data are presented as number (%). SNP: single nucleotide polymorphism; IL: interleukin; TNF: tumour necrosis factor; RANTES: regulated on activation, normal T-cell expressed and secreted. [#]: n=200; [¶]: n=313; [1]: two controls missing, [2]: one patient missing.

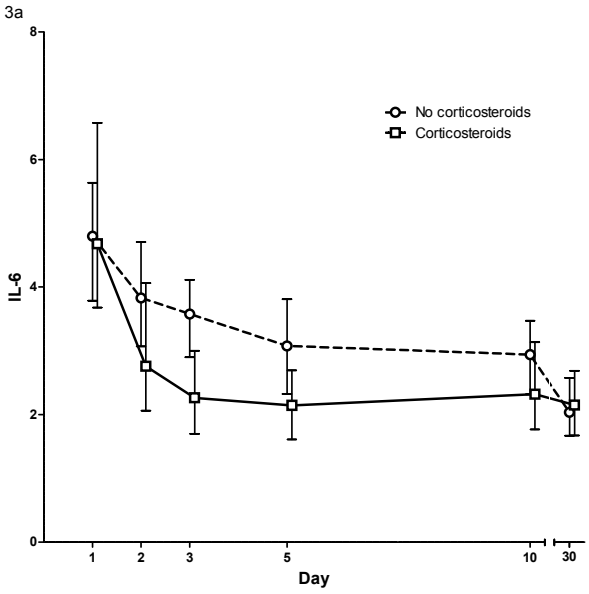
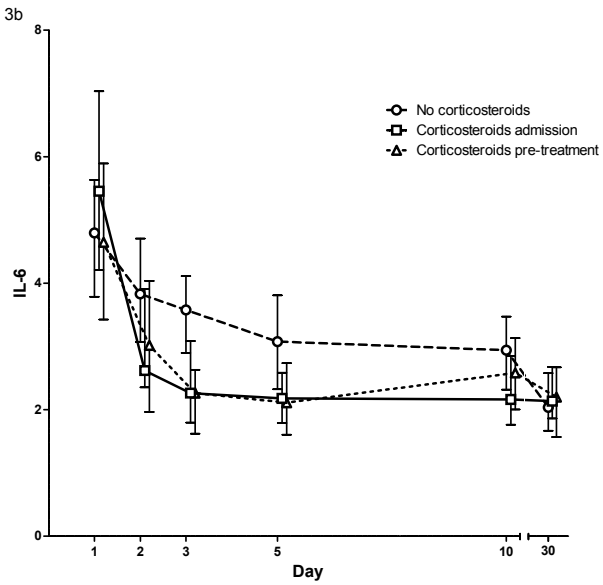



**Figure 3.** The effect of corticosteroids on interleukin (IL)-6 response.
**a**: Median IL-6 levels (ln scale) for patients on corticosteroid therapy that was started because of a pulmonary infection within 5 days prior to admission or at the ED during admission (corticosteroids) and patients who did not use corticosteroids at all (no corticosteroids) are plotted. After adjusting for age, sex, Pneumonia Severity Index score, chronic obstructive pulmonary disease, IL-6 level on day 1 and pneumococcal pneumonia, corticosteroid therapy independently influenced IL-6 levels on day 3 ($p<0.01$).




**b**: Median IL-6 levels (ln scale) for patients on corticosteroid therapy that was started because of the pulmonary infection ≤5 days before hospital admission (corticosteroid pre-treatment), patients who started corticosteroids at the time of hospital admission (corticosteroids at admission), and patients who did not use corticosteroids at all (no corticosteroids) are plotted. No statistically significant differences in IL-6 levels on admission or during hospital stay were seen between the two groups on corticosteroid therapy. In both groups IL-6 levels on day 3 were independently influenced by corticosteroids ($p<0.01$).

**Antibiotics**

We combined the causative microorganisms found in 128 patients with the antibiotics used and scored the antibiotic therapy as being either appropriate or inappropriate (see appendix C). After admission, 86% of the patients were treated with an appropriate antibiotic. At days 2, 3 and 5 this number increased to 86%, 89% and 92%, respectively. We did not find any differences in the cytokine levels between patients treated with appropriate or inappropriate antibiotics. The use of antibiotics prior to admission had no effect on the levels of cytokines, except for the serum levels of IL-6 at admission ($p=0.04$), but this effect was lost after multivariate analysis combining antibiotic therapy prior to hospitalisation, PSI score and causative microorganism.

**Corticosteroids**

Of the 201 enrolled patients, 35 patients were using a corticosteroid upon admission (mean prednisone equivalent dose 14 mg/day). In 29 patients, corticosteroid treatment was initiated on the day of admission to the hospital. In total, corticosteroid therapy (mean prednisone equivalent dose 43 mg/day) was prescribed for 62 patients after admission (in two patients corticosteroid therapy was stopped at admission). Patients who were treated with corticosteroids were significantly older (median age 72.5 versus 62 years, $p<0.01$) and more frequently known to have a history of COPD (74% versus 11%, $p<0.01$). The PSI also differed between the two groups: the median PSI score was significantly higher in patients treated with corticosteroids compared to patients who were not treated with corticosteroids (102 versus 77, $p<0.01$). Median serum IL-6 levels were similar on the day of admission, but decreased more rapidly by day 3 (at least 24 h on corticosteroids) for the corticosteroid treated group (*Figure 3a*). On days 2, 3, 5 and 10 the median IL-6 levels were significantly lower in patients treated with corticosteroids. On the day of the control visit, median IL-6 levels had returned to baseline levels and did not differ between both groups. After adjusting for age, sex, PSI score, COPD, IL-6 level on day 1, and pneumococcal pneumonia, corticosteroid therapy independently influenced IL-6 levels on day 3 ($p<0.01$, $\beta$: -1,23). Where $\exp(-1.23) = 0.29$, mean Il-6 levels were 70% less for patients using corticosteroids. Similar results were found for the IL-1Ra and MCP. IL-10 levels in patients treated with corticosteroids were significantly higher at admission. Therefore, in the multivariate analysis we found no significant effect of corticosteroid therapy on IL-10 levels on day 3. There was no effect of corticosteroid treatment on ICU admission or mortality.

Patients, not receiving corticosteroids on a chronic basis, could be divided into two groups who either started with corticosteroids ≤5 days before admission or who started corticosteroids after admission (*Figure 3b*). Age, sex, PSI and

history of COPD did not differ between these groups. There were no differences in the IL-6 levels at admission or kinetics of the response between the two groups. There were no differences in the IL-6 levels at admission or kinetics of the response between patients who started corticosteroids ≤5 days before admission or who started corticosteroids after admission. Both groups had a significant lower IL-6 level on day 3 compared to patients without the use of corticosteroids.



## Discussion

We report a comprehensive analysis of the systemic cytokine response during the clinical course of CAP in relation to cytokine polymorphisms, causative microorganisms, antibiotics, use of corticosteroids and clinical course. The major findings of this study are that in patients with CAP, serum IL-6, IL-8, IL-1Ra, and IL-10 act as acute phase proteins. After admission, the levels of these cytokines decreases rapidly, but SNPs in the IL-6, IL-8, and IL-10 genes (including haplotypes) do not influence cytokine production, and no association was found between cytokine polymorphisms and clinical outcome. Distribution of cytokine polymorphisms did not differ between patients (with different causative microorganisms) and healthy volunteers. IL-6 and IL-1Ra levels are significantly higher at admission in patients with pneumococcal pneumonia. Furthermore, the decrease in levels of IL-6, IL-1Ra, and MCP on consecutive days was independently influenced by the start of corticosteroid therapy. IL-6 and IL-1Ra levels are significantly higher at admission in patients with pneumococcal pneumonia.

Next to CRP, IL-6, IL-1Ra, IL-10 and IL-8 were identified as acute phase proteins. No acute phase response was observed for IL-5, IL-12, IFN-γ, MCP and MIP. Our results are comparable with former findings in human and experimental pneumococcal pneumonia, in which the inflammatory response is characterised by an extended pro-inflammatory response resulting in high levels of IL-6 and IL-1Ra.[2,9,14,21,35-37]. In this study, cytokines and chemokines were only measured in serum. It might be possible that a number of cytokines, and especially chemokines, are up-regulated in the lung itself, but not in the systemic circulation.[15] Our results are limited to systemic inflammatory response as measured by serum cytokines.

Admission to the hospital marks initiation of treatment by administration of intravenous antibiotics in nearly all patients. In 86% of the patients with a known causative microorganism, an appropriate antibiotic therapy, suitable for eliminating the causative microorganism, was started in our study. Effective tissue concentrations were assured by administering the antibiotics intravenously, so this is the most probable explanation for the decrease in

cytokines. The sharp decrease in IL-6, IL-8, IL-1Ra, and IL-10 within the first 2 days of admission is in contrast with the more blunt kinetics of the CRP response. In daily practice, CRP is usually regarded as the most important acute phase protein in CAP.

It should be noted that the dynamic changes in IL-6 occur earlier than CRP.

In experimental in vitro response to *S. pneumoniae*, T-helper cell (Th) type 1 cytokine production (IL-12, INF-γ) was observed in the early phases of the disease.[38-41] However, this was not observed in patients, suggesting that Th1 cytokines do not participate in the acute phase response in human (pneumococcal) pneumonia. Alternatively, the putative Th1 response could occur in the very early phase of disease, and levels of these cytokines returned to normal by the time of presentation to the ED.

The lack of finding a difference in distribution of cytokine polymorphisms between patients and controls could be that other patient characteristics are far more important in susceptibility and clinical course of CAP, such as age, medical history and causative microorganism. For the latter, it is known that CAP caused by a specific microorganism is associated with cytokine polymorphism.[25,29,42] Although we were able to identify causative microorganism in 64% of the patients, the groups of patients with a single causative microorganism were too small for further analysis.

In healthy volunteers, polymorphisms in cytokine encoding genes determine the magnitude of the endotoxin induced systemic cytokine response[43-45] We found no effect of genetic polymorphisms in cytokine genes on the ultimate levels or kinetics of the cytokine response in CAP, probably due to an overwhelming inflammatory response caused by a high amount of microorganisms, endotoxins and other pro-inflammatory proteins. In healthy volunteers, the cytokine response is stimulated by small doses of endotoxins, and without living microorganisms or other pro-inflammatory proteins. This might explain the polymorphism-dependent differences in cytokine responses observed in healthy volunteers, but not in patients.

*Post-hoc* analysis revealed a significant effect of the start of corticosteroid therapy on the levels of IL-6, IL-1Ra, and MCP (corrected for possible confounders). This decrease is most probably due to the anti-inflammatory properties of the corticosteroids and is in agreement with previous literature.[46,47] Although, this may also be due to the effect of upregulated regulatory cytokines. IL-10 levels were higher at admission in patients on pre-hospital corticosteroid therapy compared to patients who weren't. The majority of this group of patients (74%) suffered of COPD, which is regarded as a chronic inflammatory disease. It is possible that during the early phase of CAP, regulatory cytokines are already in an activated state in patients with COPD on corticosteroid therapy.[48,49] Patients treated with corticosteroids shortly before admission showed the same

decrease in IL-6 levels compared to patients who started corticosteroids at admission. This suggests that a low dose of corticosteroids may not be sufficient to decrease cytokine levels. Prospective, larger scale trials are warranted to evaluate the effect of corticosteroids on cytokine kinetics and outcome.

In pneumococcal pneumonia, IL-6 and IL-1Ra play a more prominent role in the innate immune response than in CAP caused by other microorganisms. One reason could be that *S. pneumoniae* is an extracellular pathogen and causes a more invasive disease.[50] A number of the other causative pathogens are intracellular bacteria and therefore the nature of the cytokine response will differ.[51,52] An alternative, but not mutually exclusive explanation is that the clinical course of pneumococcal pneumonia is more rapid and severe; patients would therefore present themselves at the hospital shortly after or at the peak of the cytokine storm. However, we did not find differences in the length of the period between the first clinical signs until hospital admission in pneumococcal and nonpneumococcal patients.

Inadequate antibiotic treatment may influence the kinetics of the cytokine response. The causative pathogen was identified in 64% of the patients and in the majority of the patients adequate treatment was given. Inadequate treatment was initially only given to patients with atypical bacteria, such as *M. pneumoniae*. These patients normally have a mild disease probably with a modest cytokine storm and therefore the results will be mildly influenced.

The main limitations of this study are the different nature of the causative microorganisms, the lack of a validated clinical score to reflect severity of disease during hospital stay and a lack of power to predict clinical outcome. As shown, the cytokine response differs in patients dependent of the causative microorganism of CAP. Relationships between cytokine levels, polymorphisms and clinical characteristics could be clearer in a group of patients with one causative microorganism. Our study is underpowered to perform analysis in subgroups (for example, patients with pneumococcal pneumonia). Another limitation is the lack of a validated score system for severity of disease. As we were interested in the level of cytokine response in relation to severity of disease. PSI at admission is a score to predict mortality and appeared not to be useful a as score for severity of disease during hospital stay. Finally, due to a low mortality rate and few ICU admissions we were unable to show an association between cytokine levels and outcome.

In conclusion, we show that IL-6 and IL-8, together with IL-1Ra and IL-10 act as acute phase proteins in CAP; IL-6 is a more accurate biomarker for the follow-up of CAP than CRP. Genotype does not influence the levels of cytokine production in CAP. Furthermore, the systemic cytokine response is influenced by the causative microorganism and the cytokine response can be reduced by the use of corticosteroids in patients with CAP.

## Appendix A

Each sample was drawn into pyrogen-free vials, and, within 2 hours, the serum was separated by centrifugation and stored at −80 °C. Cytokine concentrations were determined by multiplex immunoassay using a 10-plex human cytokine kit from BioRad Laboratories (Hercules, CA, USA).[53,54]
The assay was performed according to the manufacturer's instructions, and was run and analysed on a Bio-Plex 100 Suspension Array System (BioRad).

## Appendix B

Genotyping was performed with TaqMan genotyping technology using the manufacturers protocol. Sequences of primers and probes are given below. Negative controls consisted of double destilated $H_2O$ added to the reaction mix and were added to the PCR plate as well.

| SNP | Primers | Probes |
|---|---|---|
| IL-6 -572 G/C (RS1800796) | GCCTTGAAGTAACTGCACGAAATT<br>CCAGTCATCTGAGTTCTTCTGTGTT | AACAGCCGCTCACAG<br>TACAACAGCCCCTCACAG |
| IL-8 -251 T/A (RS4073) | GTTCTAACACCTGCCACTCTAGTAC<br>CATTTAAAATACTGAAGCTCCACAATTTG GT | AAGCATACAATTGATAATT<br>AGCATACATTTGATAATT |
| IL-10 -592 C/A (RS1800872) | CCAAGACAACACTACTAAGGCTTCT<br>GCTGGATAGGAGGTCCCTTACTTT | CCTACTTCCCCCTCCCAA<br>CCTACTTCCCCTTCCCAA |
| IL-10 -1082 G/A (RS1800896) | GCCCTTCCATTTTACTTTCCAGAGA<br>GGTAAAGGAGCCTGGAACACATC | CCCGCCTGTCCTGTAG<br>CCGCCTGTACTGTAG |
| IL-10 +3367 G/A (RS3024495) | GCAGAGTTTGATGAAAAGACATTAGAGGAA<br>TTGGTGGGAGAACACAGACATTTAA | CTCTCACCGTCTTGC<br>CTCTCACCATCTTGC |
| IL-18 -137 G/C (RS187238) | CACAGAGCCCCAACTTTTACG<br>GGCAGAGGATACGAGTACTTCTTTT | ACTATTTTCATGAAATCTTTTCT<br>TTTTCATGAAATGTTTTCT |
| TNFα -238 G/A (RS361525) | CAGTCAGTGGCCCAGAAGAC<br>CCCTCACACTCCCCATCCT | CTCGGAATCGGAGCAG<br>CTCGGAATCAGAGCAG |
| RANTES 1.1 G/A (RS2280789) | TGCTTCATGGCAGGGATCTC<br>GTGAACACCTGTAGGCCTTGAG | TTTTTCTGTCTTTAAGGTCTAC<br>CTGTCTTCAAGGTCTAC |

## Appendix C

Antibiotics in CAP defined as appropriate or inappropriate therapy according to the most frequently identified causative microorganism.

| | ***Streptococcus pneumoniae*[1]** | ***Haemophilus influenzae*[2]** | ***Legionella pneumophilia*** | ***Mycoplasma pneumoniae*** | ***Staphylo-coccus aureus*[3]** |
|---|---|---|---|---|---|
| **Penicillin** | + | - | - | - | - |
| **Amoxicillin** | + | + | - | - | - |
| **Amoxicillin- clavulanate** | + | + | - | - | + |
| **Cephalosporins** | + | + | - | - | + |
| **Macrolides** | - | - | + | + | - |
| **Quinolones** | - | + | + | - | - |

+ = appropriate , – = inappropriate antibiotic therapy
[1] all *S. pneumoniae* penicillin sensitive, [2] all *H. influenzae* amoxicillin sensitive, [3] no MRSA

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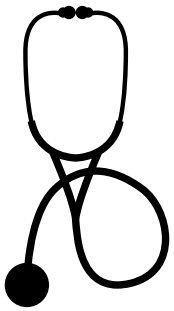

# Chapter 4

# Mannose-binding lectin (MBL2) and Ficolin-2 (FCN2) polymorphisms in patients on peritoneal dialysis with staphylococcal peritonitis

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## Abstract

### Background

Mannose-binding lectin (MBL) and ficolin-2 (FCN) are activators of the lectin pathway of complement and act as primary defences against infection. Single-nucleotide polymorphisms (SNPs) in the MBL2 and FCN2 genes influence the functionality of the proteins. Both proteins are capable of binding staphylococci, which are pathogens that frequently cause peritonitis in patients on continuous ambulatory peritoneal dialysis (CAPD). We studied the role of polymorphisms in the MBL2 and FCN2 genes as a risk factor for developing CAPD peritonitis caused by staphylococci.

### Methods

We analysed SNPs in the MBL2 and FCN2 genes in 40 CAPD patients with staphylococcal peritonitis and in 65 CAPD patients without any history of peritonitis. Additionally, we analysed the prevalence of exit site infections and nasal *Staphylococcus aureus* carriage in both groups.

### Results

The +6359C>T SNP leading to the Thr236Met amino acid alteration in the FCN2 gene, associated with decreased substrate binding, was significantly more present in CAPD patients with a history of staphylococcal peritonitis compared with patients on CAPD without a history of peritonitis (p=0.037). No difference was found in MBL2 genotypes between the two groups. In CAPD patients with a history of staphylococcal peritonitis, exit site infection with *S. aureus* was also more prevalent (p<0.01), while *S. aureus* carriage was not (p=0.073)

### Conclusion

In addition to known risk factors such as exit site infection, the +6359C>T SNP in the FCN2 gene might be a risk factor for staphylococcal peritonitis in CAPD patients due to decreased binding of FCN to staphylococci.

## Introduction

Patients with end-stage renal disease have an increased susceptibility to infection, partly due to the impaired immunity caused by uraemia. In patients on continuous ambulatory peritoneal dialysis (CAPD), peritonitis is a common complication with significant morbidity and mortality. The most commonly found causative microorganism is *Staphylococcus aureus*. Staphylococcal peritonitis is caused by local spreading from the skin via the catheter. Furthermore, nasal carriage of *S. aureus* is a known risk factor for *S. aureus* peritonitis. The role of intrinsic factors (e.g., the patient's immune system) is a subject of research.

Mannose-binding lectin (MBL) is a calcium-dependent C-type lectin that acts as a primary defence mechanism against infections. MBL is an activator of the complement system and enhances phagocytosis. MBL deficiency may confer a risk of infection, especially when other mechanisms of immunity are impaired. MBL is capable of binding to a broad range of microorganisms and has a strong binding capacity for *S. aureus*.[1,2] Ficolin-2 (FCN) is a serum protein that is similar to MBL in structure and function. After binding microorganisms, it can activate complement and enhance phagocytosis by opsonisation. FCN can recognise *S. aureus* by binding lipoteichoic acid (LTA) moieties on the bacterial surface.[3]

Functional MBL levels are influenced by single-nucleotide polymorphisms (SNPs) in exon 1 and the MBL2 gene promoter region. The combination of these SNPs results in sufficient or deficient MBL serum levels.[4] Polymorphisms in the FCN gene FCN2 have been described.[5,6] Genetic differences in the 5'untranslated region (5'-UTR) influence FCN levels and two coding SNPs in the fibrinogen-like domain alter the substrate binding affinity.[7-9]

In CAPD patients, MBL2 and FCN2 gene SNPs may influence defences against staphylococci, since the overall immunity is already impaired. This might lead to an increased risk to staphylococcal peritonitis. The aim of this study was to examine whether polymorphisms in the genes encoding MBL and FCN act as risk factors for staphylococcal peritonitis in CAPD patients.

## Methods

### Patients and controls

In this retrospective study we used information from databases of all continuous ambulatory peritoneal dialysis (CAPD) patients in two centres from January 1997 through August 2009. Subjects were included in the study if they were at least 18 years old and were on CAPD for more than 6 months. Staphylococcal peritonitis was defined as a white blood cell count in PD fluid >100/mm$^3$, with PD fluid cultures that were positive for staphylococcal species (*S. aureus* or coagulase-

negative staphylococci). Relapsing peritonitis, defined as the development of peritonitis caused by the same microorganism within 2 weeks, was regarded as the same peritonitis episode. Using these criteria, 40 patients with at least one episode of staphylococcal CAPD peritonitis and 65 control patients without any history of peritonitis were included. In the peritonitis group, 32 patients were asked to donate whole-blood samples for DNA analysis. Of eight peritonitis patients who were deceased, serum samples were available for analysis. In the 65 control patients, 45 patients donated whole blood, and from 20 patients serum samples were available. Exit site infection was defined as colonisation of the PD catheter exit site with staphylococcal species and clinical signs of inflammation (e.g. redness of the skin). Nasal *S. aureus* carriership was defined by at least two positive nasal cultures with *S. aureus*. A previously described second control group consisted of 223 Caucasian blood donors, not on CAPD, who originated from the same geographical area as the patients.[10,11] Written informed consent was obtained from all living patients. The study protocol was approved by the institutional medical ethics committee.

**DNA isolation**

Genomic DNA was isolated from whole-blood samples obtained from 76 patients. To increase the number of patients available for genotypic analysis, genotyping from previously stored serum samples from 29 patients was used. Genomic DNA was isolated from 100 µL of whole blood or serum with the MagNAPure LC robot (Roche Diagnostics, Mannheim, Germany), using the MagNAPure DNA Isolation Kit according to the manufacturer's protocol.

**Genotyping of MBL2**

The X/Y promoter (rs7096206) and exon 1 SNPs (wild-type 'A' and variants 'O' rs5030737, rs1800450 and rs1800451) of MBL2 were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay with modifications in a nested PCR protocol.[12,13] Two PCR assays specific for the promoter X or Y SNP were run in a nested PCR assay. For whole-blood samples, the PCR was run for 25 cycles as previously described.[14] For serum samples, it was run for 40 cycles. After a 1:100 dilution in PCR-grade water (Sigma-Aldrich, Zwijndrecht, the Netherlands), MBL2 exon 1 was amplified from these PCR products with an additional GC clamp attached to one primer to meet DGGE requirements. Amplified DNA fragments from the second PCR assay were analysed on a polyacrylamide gel with a linear denaturing gradient of formamide and urea. All MBL2 exon 1 haplotypes could be distinguished by their different patterns of migration. Genotypes YA/YA, XA/YA, XA/XA and YA/O were considered 'MBL-sufficient', and genotypes XA/O and O/O were considered 'MBL-deficient'.[15-17]

### Genotyping of FCN2

Three FCN2 genotypes were determined using a previously described DGGE assay[18]: one SNP in the 5'-UTR (-4A>G, rs17514136, associated with elevated FCN serum levels) and two coding SNPs in exon 8 (+6359C>T, rs17549193, associated with decreased substrate binding and +6424G>T, rs7851696, associated with increased substrate binding). For DNA isolated from serum samples, this protocol was modified to incorporate a nested PCR assay. An initial PCR for exon 1 (forward primer TCG GAA GAT GAG AAA TTG G, reverse primer CAG GGA CGA GAA GTT TCC) and exon 8 (forward primer CCT GCC TAA CCA TAC ATG G, reverse primer AAC AGA GCT GGA TTT GAA CC) was performed (annealing for 60 s at 57°C, 40 cycles). A 1:100 dilution of this PCR product served as the template for further amplification in the whole -blood genotyping assay as described above.



### Statistical analyses

All statistical analyses were performed using statistical software (SPSS version 15.0 for Windows, Chicago). The contributions of polymorphisms in the MBL2 and FCN2 genes and clinical characteristics (e.g. age, sex, exit site infection, nasal *S. aureus* carrier status) to the development of CAPD staphylococcal peritonitis were analysed using univariate analysis (Pearson's Chi-square test or the Fisher's exact test, as appropriate). Statistical significance was reached at the p=0.05 level. All polymorphisms adhered to the Hardy-Weinberg expectations (p>0.05).

## Results

### Baseline characteristics

There were no significant differences in age, sex or mean time on CAPD between patients and controls (*Table 1*). Nasal *S. aureus* carriage was not measured in one of the centres. Therefore carriage was scored in 56 patients only. Nasal *S. aureus* carriage was equally prevalent between both groups (p=0.073). Significantly more patients with a history of peritonitis had one or more exit site infections with *S. aureus*, as compared with the CAPD patients without a history of peritonitis (p<0.01).

**Table 1.** Baseline characteristics

| | **Patients with a history of staphylococcal peritonitis n = 40** | **Patients without a history of peritonitis n = 65** | **p-value** |
|---|---|---|---|
| **Age, years** | 53 (18) | 59 (13) | NS |
| **Sex, male** | 23 (58) | 38 (58) | NS |
| **Time on PD, months** | 37 (22) | 30 (23) | NS |
| **Number of staphylococcal peritonitis episodes** | 1.58 (1-5) | - | |
| **Causative agent of peritonitis** | | | |
| *S. aureus* | 20 (50) | - | |
| CNS | 20 (50) | - | |
| **Exit site infection with *S. aureus*** | 22 (55) | 12 (19) | <0.01[a] |
| **Nasal *S. aureus* carrier[b]** | 11/20 (55) | 11/36 (31) | NS |

Date are number (%), mean (SD) or mean (range)
[a]: Statistically significant .
[b]: Due to unavailable nasal cultures, the denominator has been changed.

## Genotype distribution

No differences were seen in MBL2 genotype distribution (sufficient vs. insufficient) between CAPD patients who had a history of staphylococcal peritonitis and patients without a history of peritonitis (*Table 2*; Pearson's Chi-square, p=0.240). Eight percent of the patients with CAPD peritonitis had a genotype coding for deficient MBL production (O/O or XA/O), compared with 17% of the patients without peritonitis. This distribution did not differ from that of healthy individuals (data not shown).

The +6359C>T SNP in the FCN2 gene, associated with decreased substrate binding, was significantly more present in patients with a history of staphylococcal peritonitis compared with patients on CAPD without a history of peritonitis (*Table 3*; 18% vs. 5% respectively, Pearson's Chi-square p=0.037). Moreover, there is a tendency towards a gene dose effect. Patients with genotype +6359T/T had more frequent peritonitis than patients with genotype +6359C/C [odds ratio (OR) = 5.57, 95% CI 1.29 – 24.05, p=0.013]. There is a trend that in patients with genotype +6359T/T peritonitis was more frequent than in the patients with the +6359C/T genotype (OR = 3.05, 95% CI 0.66 – 14.14, p=0.144). The OR for genotype +6359C/T compared to genotype +6359C/C is 1.83 (95% CI 0.74 – 4.53, p=0.191). No differences were seen for the other two SNPs in the FCN2 gene.

**Table 2.** MBL genotypes.

| | Patients with a history of staphylococcal peritonitis n = 40 | Patients without a history of peritonitis n = 65 | p-value |
|---|---|---|---|
| **MBL2 genotype** | | | NS* |
| **MBL-sufficient** | 37 (92) | 54 (83) | |
| A/A | 27 (68) | 30 (46) | |
| XA/XA | 0 (0) | 3 (5) | |
| YA/O | 10 (25) | 21 (32) | |
| **MBL-deficient** | 3 (8) | 11 (17) | |
| XA/O | 2 (5) | 6 (9) | |
| O/O | 1 (3) | 5 (8) | |

Data are number (%). AA/CC/GG, wild-type homozygous; AG/CT/GT, wild-type/variant-type heterozygous; GG/TT, variant-type homozygous. *p=0.240, MBL-sufficient vs. MBL-deficient.

**Table 3.** FCN genotypes.

| | Patients with a history of staphylococcal peritonitis n = 40 | Patients without a history of peritonitis n = 65 | p-value |
|---|---|---|---|
| **FCN2 genotype** | | | |
| **-4A>G[a]** | | | NS |
| A/A | 20/37 (54) | 42/63 (67) | |
| A/G | 13/37 (35) | 18/63 (29) | |
| G/G | 4/37 (11) | 3/63 (5) | |
| Allele frequencies | | | |
| A allele % | 72 | 81 | NS |
| G allele % | 28 | 19 | |
| **+6359C>T[a]** | | | 0.037[b] |
| C/C | 18/38 (47) | 43/63 (68) | |
| C/T | 13/38 (34) | 17/63 (27) | |
| T/T | 7/38 (18) | 3/63 (5) | |
| Allele frequencies | | | 0.006[b] |
| C allele % | 64 | 82 | |
| T allele % | 36 | 18 | |
| **+6424G>T[a]** | | | NS |
| G/G | 28/38 (74) | 45/63 (71) | |
| G/T | 10/38 (26) | 17/63 (27) | |
| T/T | 0/38 (0) | 1/63 (2) | |
| Allele frequencies | | | |
| G allele % | 87 | 85 | NS |
| T allele % | 13 | 15 | |

Date are number (%). AA/CC/GG, wild-type homozygous; AG/CT/GT, wild-type/variant-type heterozygous; GG/TT, variant-type homozygous.
[a]: Due to missing values, the denominator has been changed. [b]: Statistically significant

## Discussion

In this cohort of 105 patients on CAPD, the +6359C>T SNP leading to the Thr236Met amino acid alteration in the FCN2 gene was associated with an increased risk of developing staphylococcal peritonitis in patients on CAPD. SNPs in the MBL2 gene were not associated with an increased risk of staphylococcal peritonitis. Significantly more patients with a history of peritonitis had one or more exit site infections with *S. aureus*, compared with the control patients without peritonitis.

FCN levels and polymorphisms in the FCN-2 gene have been associated with a variety of infectious diseases and autoimmune diseases.[19-22] Several SNPs have been demonstrated to influence FCN serum levels as well as ligand binding affinity.[23,24] Furthermore, low levels of FCN may contribute to susceptibility of respiratory infections.[25]

The +6359C>T SNP in the FCN2 gene is associated with decreased ability of carbohydrate binding, but it has no effect on FCN levels.[26,27]

Several SNPs in the promoter region of FCN2 have been associated with susceptibility for rheumatic fever and Behcet's disease.[28,29]

CAPD has been associated with lower serum MBL levels, compared with healthy controls.[30] These lowered MBL levels in patients on CAPD were not associated with exon 1 SNPs but were proposed to be due to MBL loss via peritoneal clearance and reduced MBL synthesis in the liver due to uraemia.[31] A decreased MBL level may be an underlying risk factor for infections in patients on peritoneal dialysis. On the other hand in haemodialysis patients MBL levels were significantly higher.[32] In a subsequent study it was suggested that lower MBL levels predict unfavourable outcome in haemodialysis patients.[33]

Besides exon 1 SNPs, the promoter X/Y SNP greatly influences MBL levels. The genotype YA/O is considered to be MBL-sufficient, while XA/O is considered to be MBL-deficient.[34] Furthermore, genotype XA/XA is not able to upregulate production when MBL is consumed (e.g. during the acute phase of infection).[35] Our data suggest that the differences in distribution of X/Y promoter SNPs is not responsible for staphylococcal infections in CAPD patients.

In a previous study, peritonitis in CAPD patients was not associated with peritoneal MBL levels or exon 1 SNPs of MBL2.[36] However, in that study, all causes of peritonitis were considered, including Gram-positive and Gram-negative pathogens, mixed flora and culture-negative peritonitis. Furthermore, the study was limited to MBL only as the activating molecule of the lectin pathway of complement. Our study specifically looked at the association of staphylococcal peritonitis with genetic variations in two initiators of the lectin pathway, MBL and FCN; both have a strong affinity for staphylococci. In our cohort, MBL genetic variations did not explain why some CAPD patients had

staphylococcal infections and why some CAPD patients did not have peritonitis at all.
Other predisposing risk factors for peritonitis are also of importance (i.e. exit site infections, connecting technique of the catheter, nasal *S. aureus* carrier status, personal hygiene).[37,38] Also in our study, we observed an increased prevalence of exit site infection in those who developed staphylococcal peritonitis.
The limited sample size is a potential weakness of the study. In this relatively small study population we did not observe any effect of MBL on staphylococcal peritonitis. We therefore consider major effects of MBL genotype on the occurrence of staphylococcal peritonitis unlikely. However, we did find a significant difference in the +6359C>T SNP of the FCN2 gene. Larger study populations are needed to investigate potential weaker effects in the other SNPs.

In conclusion, the +6359C>T SNP in the FCN2 gene might be a risk factor for staphylococcal peritonitis in CAPD patients due to decreased binding of variant FCN to staphylococci. This study shows that besides classical risk factors for CAPD peritonitis (e.g. exit site infection), genetic variations in the immune system can lead to an increased risk of peritonitis in patients on CAPD.

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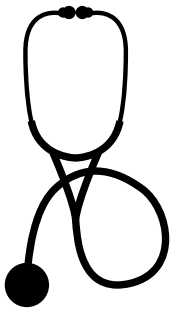

# Chapter 5

# Lectin mediated complement activation by *Streptococcus pneumoniae*

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## Abstract

### Background

The role of MBL and ficolin-2 in the opsonisation of pneumococci is considered to be of limited importance. In the few published *in-vitro* studies little or no binding of MBL and ficolin-2 was found on the surface of pneumococci. However, MBL deficient patients have a substantially increased risk of invasive pneumococcal disease. The aim of this study is to investigate the binding of MASP-2 to pneumococci and whether eventual binding of MASP-2 results from binding of MBL and/or ficolin-2.

### Methods

In 32 patients with community-acquired pneumonia, *S. pneumoniae* was isolated and serotyped. All patients were genotyped for MBL and ficolin-2 polymorphisms. Using flow cytometry analyses, binding of MBL, ficolin-2 and MASP-2 on the surface of the pneumococci was measured after incubation with homologous serum.

### Results

MASP-2 binding was found in 19/32 patients (59%). MBL binding was found in 13/32 patients (41%) and ficolin-2 binding in 28/32 patients (88%). MASP-2 binding was independent of *S. pneumoniae* serotype. In a subgroup of patients in which binding MBL but no ficolin-2 on the pneumococci was observed, no significant MASP-2 binding was seen.

### Conclusion

In this study we show that pneumococci can bind MASP-2. Ficolin-2 seems to be the overriding MASP-2 activator. The data suggest a serotype independent activation of the lectin pathway of complement by pneumococci.

## Introduction

Community-acquired pneumonia (CAP) is common, and it remains the leading cause of infection worldwide. In approximately 50 percent of all cases, CAP is caused by *S. pneumoniae*.[1] *S. pneumoniae* is a Gram-positive coccus with a polysaccharide cell capsule. There are 90 serotypes of *S. pneumoniae* that are differentiated by the capsular polysaccharide coat, which protects the pneumococci from phagocytosis by polymorphonuclear leukocytes. A serotype consists of several strains.

There are different routes of complement activation: the classical, the alternative and the lectin pathway. The lectin pathway is based on the recognition of carbohydrates, such as D-mannose, glucose, N-acetyl-glucosamine (GlucNAc) and N-acetyl-mannosamine.[2,3] Mannose binding lectin (MBL) is a serum lectin that is produced by the liver and binds mannose and several carbohydrate structures that are present on the surfaces of a diverse set of microorganisms. After binding, MBL activates the lectin pathway of complement, and it mediates opsonophagocytosis of the microorganism. Ficolin-2 is a serum protein that is similar to MBL in structure and function. After binding microorganisms, MBL and ficolin-2 activate the lectin pathway of complement in an antibody-independent manner. In this lectin pathway, MBL-associated serine protease-2 (MASP-2) can be bound by MBL and ficolin-2. After binding, MASP-2 becomes enzymatically active and cleaves first C4 and C2 to form the C3 convertase, C4b2a. This enzyme generates significant amounts of opsonic C3b fragments that coat microorganisms for phagocytosis.

The levels and functional activity of MBL and ficolin-2 are respectively influenced by single nucleotide polymorphisms (SNPs) in exon 1 and the promoter region of the MBL2 gene and genetic differences in the 5' untranslated region (5'-UTR) and two coding SNPs in the fibrinogen-like domain of the FCN2 gene.

MBL plays only a minor role in the opsonisation of *S. pneumoniae*.[4,5] Direct *in-vitro* demonstration of MBL binding to pneumococci is limited, and no differences in the phagocytosis of pneumococci are seen between MBL sufficient and MBL deficient patients.[6] In contrast, MBL deficient patients do have a substantially increased risk of invasive pneumococcal disease.[7-9] The role of ficolin-2 in the opsonisation of pneumococci is unclear. Ficolin-2 binds some Gram-positive bacteria, but it only binds to 2 of 20 serotypes of *S. pneumoniae*.[10] Moreover the contribution of ficolin-2 to the opsonophagocytosis is also limited.[11]

The aim of this study was to investigate the binding of MASP-2 to pneumococci and determine whether the eventual binding of MASP-2 resulted from the binding of MBL and/or ficolin-2. Furthermore, we evaluated the role of pneumococcal serotype and MBL/ficolin-2 genotype on the activation of the lectin pathway of complement binding.

## Methods

### Study population

Data were obtained from patients hospitalised with CAP who participated in two consecutive clinical trials.[12,13] Patients were hospitalised in the period October 2004 to August 2006 and November 2007 to September 2010 in a general 600-bed teaching hospital in the Netherlands. In- and exclusion criteria are described in more detail elsewhere.[13] CAP was defined as a new infiltrate on a chest radiograph in combination with at least two of the following criteria: cough, sputum production, temperature >38°C or <35°C, auscultatory findings consistent with pneumonia, C-reactive protein (CRP) >15 mg/l, or white blood cell count of>10x109/L cells per L or <4x109/L, or >10% of rods in leukocyte differentiation.[14] Patients with defined immunodeficiencies (a known congenital or acquired immunodeficiency, chemotherapy within the last 6 weeks, oral corticosteroids use in the last 6 weeks, immunosuppressive medication in the last 6 weeks) or haematological malignancies were excluded. Patients who needed immediate admission to the intensive care unit (ICU) at presentation and pregnant or breast feeding women were also excluded.

### Serum samples and pathogen identification

Serum samples used for the analyses were obtained on the day of admission to the hospital, before any treatment was given, and were stored at -80 °C until tested. In all patients an extensive diagnostic protocol was performed to identify the causative pathogen.[15] All *S. pneumoniae* isolates from blood or sputum were serotyped by the Quellung reaction.

### Reagents

Monoclonal antibodies against human ficolin-2 (GN5), human MBL (3E7), human MASP-2 (8B5) and C4d (12D11) were obtained from Hycult Biotech, Uden, the Netherlands. Streptavidin-Phycoerythrin (SA-PE) was obtained from Bio-Rad, Veenendaal, the Netherlands. Phycoerythrin conjugated goat anti-mouse IgG1 was obtained from Jackson ImmunoResearch (West Grove, PA, USA).

### DNA isolation

Genomic DNA was isolated from whole-blood samples obtained from patients. Genomic DNA was isolated from 200 µL of whole blood with the MagNAPure LC robot (Roche Diagnostics, Mannheim, Germany), using the MagNAPure DNA Isolation Kit according to the manufacturer's protocol.

**Genotyping of MBL2**

The X/Y promoter (rs7096206) and exon 1 SNPs (wild-type 'A' and variants 'O' rs5030737, rs1800450 and rs1800451) of MBL2 were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay.[16] Briefly, two PCR assays with forward primers specific for the promoter X (ATT TGT TCT CAC TGC CAC C) or Y (TTT GTT CTC ACT GCC ACG) and a reverse primer (GAG CTG AAT CTC TGT TTT GAG TT) were run with an annealing temperature of 60 °C for 40 cycles. Amplified DNA fragment were separated and analysed on a 1.5% agarose gel. MBL2 exon 1 was amplified using a forward primer with a GC-clamp (ccg ccc gcc gcg ccc cgc gcc cgg ccc gcc gcc ccc gcc cc TCC ATC ACT CCC TCT CCT TCT C) and a reverse primer (GAG ACA GAA CAG CCC AAC ACG) in 40 cycles with an annealing temperature of 57 °C. Amplified DNA fragments were run overnight on a 6% polyacrylamide gel with a linear denaturing gradient of formamide and urea increasing from 35% to 55%. All MBL2 exon 1 haplotypes could be distinguished by their different patterns of migration. Genotypes YA/YA, XA/YA, XA/XA and YA/O were considered 'MBL-sufficient', and genotypes XA/O and O/O were considered 'MBL-deficient'.[17-19]

**Genotyping of FCN2**

Three FCN2 genotypes were determined using a previously described DGGE assay[20]: one SNP in the 5'-UTR (-4A>G, rs17514136, associated with elevated FCN serum levels) and two coding SNPs in exon 8 (+6359C>T, rs17549193, associated with decreased substrate binding and +6424G>T, rs7851696, associated with increased substrate binding).

**Measurements ficolin-2 in serum**

Ficolin-2 levels were assayed by ELISA technique (Hycult Biotech, Uden, the Netherlands). Ficolin-2 was captured using GN4 on the solid phase and detected using Streptavidin-peroxidase biotinylated GN5. Absorbances were read at A450 with a spectrophotometer. Samples were diluted at 1:25 for both serum alone or after incubation with *S. pneumoniae* (see below). Ficolin-2 levels were calculated by the standard curve based on samples with known ficolin-2 concentration.

**Detection of MBL, ficolin-2, MASP-2 and C4d deposition on the pneumococci.**

Pneumococci were grown for 6 hours at 37 degrees in Brain Heart Infusion medium, washed and resuspended at a concentration of $1x10^9$ phosphate buffer. Subsequently pneumococci were incubated for 30 minutes on ice with in 150 µl freshly thawed serum. Next pneumococci were washed by centrifugation for 3 minutes at 10,000xG. For ficolin-2 detection, $1x10^9$ pneumococci were

incubated with 25 µl of a 1:10 diluted GN5, incubated for 30 minutes on ice, washed (2x), followed by 30 minutes incubation on ice and in the dark with a 1:100 diluted goat-anti-mouse IgG PE and washed (2x). For MBL, pneumococci were incubated with 25 µl of a 1:10 diluted fluorescein isothiocyanate (FITC) conjugated polyclonal 3E7, incubated for 30 minutes on ice and in the dark and washed (2x). For MASP-2, pneumococci were incubated with 25 µl of a 1:10 diluted biotinylated 8B5, incubated for 30 minutes on ice and in the dark with SA-PE and washed (2x). For C4d, pneumococci were incubated with 25 µl of a 1:10 diluted 12D11, incubated for 30 minutes and washed (2x), followed by 30 minutes incubation on ice of a 1:100 diluted goat-anti-mouse IgG PE on ice and in the dark and washed (2x). Pneumococci were analysed by flow cytometry on a FACS Calibur (Becton Dickinson, San José, CA, USA). Each data file contained 5000 cells which were analysed by FlowJo (TreeStar, Ashland, OR, USA). For MBL and ficolin-2 an increase of median fluorescence intensity of 50% compared to a control sample (pneumococci incubated with saline) was considered to discriminate between positive and negative binding to pneumococci. For MASP-2 an increase of more than 20% was considered relevant (*Figure 1*).

## Results

A total of 505 patients were included in the two clinical trials. In 132 (26%) of the patients *S. pneumoniae* was found to be the causative pathogen. In 32 (24%) of these patients the *S. pneumoniae* bacteria was cultured and stored and serum samples were available for analyses (*Table 1*). The most prevalent serotypes were serotype 1 (n=7), serogroup 9 (n=7), serotype 7 (n=4) and serotype 14 (n=4) (*Table 1*). 3/32 patients (9.4%) were MBL deficient (XA/O and O/O). The distribution of the ficolin-2 genotypes is shown in *table 2*.

**Table 1.** Baseline characteristics of the evaluated patients

| | ***S. pneumoniae* isolated and serotyped n=32** |
|---|---|
| **Sex, male** | 22 (72) |
| **Age, years** | 57.1 (21.7) |
| **Race*** | |
| Caucasian | 31 (97) |
| Other | 1 (3.2) |
| **Comorbidities** | |
| Neoplastic disease | 3 (9.4) |
| Congestive heart failure | 4 (13) |
| Renal disease | 4 (13) |
| Diabetes mellitus | 3 (9.4) |
| COPD | 4 (13) |
| **Physical examination findings** | |
| Temperature (˚C) | 38.4 (1.1) |
| Systemic blood pressure (mm Hg) | 128.8 (21.5) |
| Heart rate (beats per min) | 106.4 (18.2) |
| Respiratory rate (breaths per min) | 26.9 (7.2) |
| **Laboratory parameters** | |
| C-reactive protein (mg/l) | 302.2 (183.4) |
| White blood count (x $10^9$/L) | 17.5 (9.3) |
| **Pneumonia severity index risk class,** | |
| Class I-III | 17 (53) |
| Class IV-V | 15 (47) |
| **Outcome** | |
| Mortality | 3 (9.4) |
| ICU admission | 4 (13) |

Data presented as mean (SD) or number (%)
*: Race was reported by the patient



## Depletion of ficolin-2 from serum by pneumococci

After incubation of serum with an excess of the *S. pneumoniae* bacteria (serum and bacteria from the same patient), ficolin-2 levels in the serum decreased. The mean overall decrease was -27% (±17.9%) but with a wide range from 0% to -65%. Patients with ficolin-2 genotype +6359T/T (associated with decreased substrate binding to GlucNAc) had a significantly lower decrease in ficolin-2 levels than the wild type ficolin-2 patients (-4% vs. -30%; p=0.015).

No differences in ficolin-2 depletion from serum were seen for the other two SNP's in FCN2 gene (*Table 2*). A decrease in ficolin-2 in serum can either mean binding to pneumococci or inactivation of ficolin-2. We therefore measured direct binding of ficolin-2 to pneumococci.

**Table 2.** MBL and ficolin-2 genotypes and decrease in ficolin-2 levels.

| | Patients with pneumococcal pneumoniae | Decrease in ficolin-2 levels | p-value |
|---|---|---|---|
| **MBL-2 genotype** | | | |
| **MBL sufficient** | | | |
| A/A | 19/32 (59) | | |
| XA/XA | 2/32 (6) | | |
| YA/O | 7/32 (22) | | |
| **MBL deficient** | | | |
| XA/O | 3/32 (9) | | |
| O/O | 1/32 (3) | | |
| **Total** | | | |
| sufficient | 28/32 (87) | -26% | 0.238 |
| deficient | 4/32 (13) | -38% | |
| **FCN-2 genotype** | | | |
| **-4A>G** | | | |
| A/A | 16/32 (50) | | |
| A/G | 14/32 (44) | | |
| G/G | 2/32 (6) | | |
| sufficient | 30/32 (94) | -29% | 0.135 |
| sufficient (elevated levels) | 2/32 (6) | -9% | |
| **+6359C>T** | | | |
| C/C | 18/32 (56) | | |
| C/T | 11/32 (34) | | |
| T/T | 3/32 (9) | | |
| sufficient | 29/32 (91) | -30% | 0.015 |
| deficient (decreased binding) | 3/32 (9) | -4% | |
| **+6424G>T** | | | |
| G/G | 22/32 (69) | | |
| G/T | 10/32 (31) | | |
| T/T | 0/32 (0) | | |
| sufficient | 22/32 (69) | -28% | 0.939 |
| sufficient (increased binding) | 10/32 (31) | -27% | |

Data presented as number (%)

**Binding of MBL, ficolin-2 and MASP-2 on the pneumococci**

For all patients we measured the direct binding of ficolin-2, MBL and MASP-2 on the pneumococci (*Table 3*, *Figure 1*). MASP-2 binding was found in 19/32 patients (59%). MBL binding was found in 13/32 Patients (41%) and ficolin-2 binding in 28/32 patients (88%). MASP-2 binding results either from MBL binding or ficolin-2 binding on the pneumococci. In 10 patients a relevant amount of MASP-2 binding was observed in the absence of MBL binding, but with observed ficolin-2 binding. However, in none of the patients MASP-2 binding was observed in the presence of MBL binding and absence of ficolin-2 binding. In a subgroup of patients in which binding of ficolin-2 alone or MBL and ficolin-2 binding on the pneumococci was observed, no significant MASP-2 binding was seen (9/36 patients, 25%). All patients showed C4d binding, although this does not discriminate between the classical pathway and the MBL/ficolin-2 mediated complement activation.

When we compared the decrease in ficolin-2 levels in serum with the binding of ficolin-2 on the pneumococci, only in 1 of the 4 patients without relevant binding of ficolin-2 to pneumococci a significant decrease (-35%) of ficolin-2 in serum was seen.

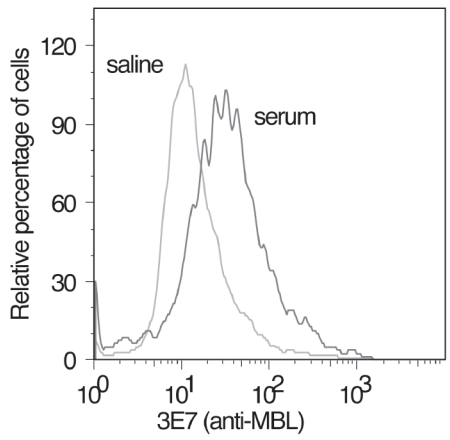
**Table 3.** Binding of MBL, ficolin-2 and MASP-2 on the pneumococci.

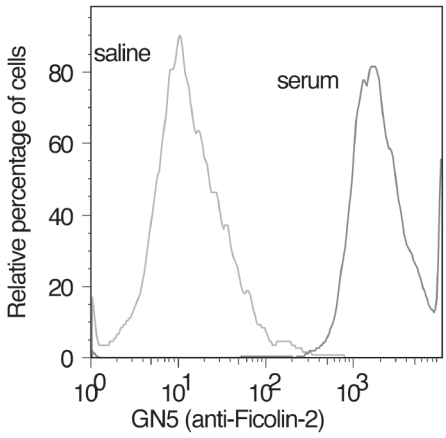
| MASP-2 binding | MBL binding | Ficolin-2 binding | Number of patients |
|---|---|---|---|
| + | + | + | 9 |
| + | – | + | 10 |
| + | + | – | 0 |
| + | – | – | 0 |
| – | – | – | 1 |
| – | – | + | 2 |
| – | + | – | 3 |
| – | + | + | 7 |

**Influence of serotype on binding**

MASP-2 binding was independent of *S. pneumoniae* serotype (*Table 4*)

The binding capacity of MBL and ficolin-2 also seemed serotype independent



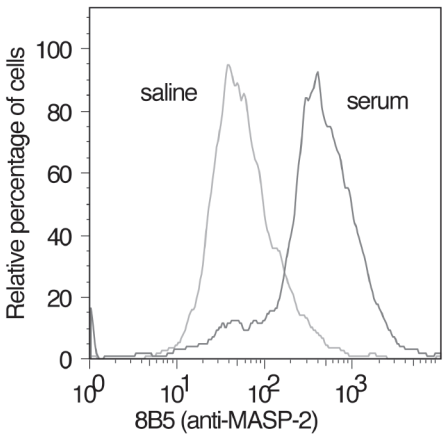




**Figure 1.** MBL , ficolin-2 and MASP-2 binding to pneumococci when incubated with serum or saline (Flow cytometry analysis)

(data not shown).

**Table 4.** MASP-2 binding for all *S. pneumoniae* serotypes.

| Serotype | MASP-2 binding n=19 | No MASP-2 binding n=13 |
|---|---|---|
| **1** | 3 | 2 |
| **3** | 0 | 1 |
| **4** | 2 | 1 |
| **6b** | 0 | 1 |
| **7** | 2 | 1 |
| **8** | 1 | 1 |
| **9** | 5 | 2 |
| **11a** | 1 | 1 |
| **14** | 2 | 2 |
| **19a** | 1 | 0 |
| **22f** | 2 | 0 |
| **23b** | 0 | 1 |



### Genotypes MBL and ficolin-2

In this cohort of patients we have 3 MBL deficient patients. All three patients did not show any significant MBL binding on the pneumococci. These three MBL deficient patients had a mean increase in ficolin-2 binding capacity of 6130% vs. 2875% in MBL sufficient patients (p=0.319).Three patients are homozygous deficient for +6359, associated with decreased binding to GlucNAc. In two of these patients this homozygous SNP for decreased binding capacity is combined with a homozygous SNP for increased ficolin-2 levels (-4G/G). Median ficolin-2 binding of these two patients was relevant (+188 and +210 % compared to the control sample), while in the patient with homozygous deficient +6359 SNP T/T and normal -4A/A, only an increase of 22% was found (considered non-relevant). However, none of the +6359 homozygous patients were able to bind MASP-2.

## Discussion

In this study we show that in a significant number of patients incubation of serum with homologous pneumococci results in deposition of MASP-2 on the bacterial surface.

In recent literature binding of MBL on pneumococci is controversial. There are clear cut clinical associations for MBL genotype and invasive pneumococcal disease (IPD).[21] On the other hand the direct *in vitro* demonstration of MBL

binding to pneumococci is limited. Several techniques have been used to detect binding of MBL and ficolin-2 to pneumococci, including depletion of MBL and/or ficolin-2 from serum after incubation with pneumococci. Krarup *et al*., using this technique, showed that MBL did not bind to 20 serotypes of *S. pneumoniae*.[22] The drawback of this technique is that high numbers of bacteria are required to deplete significant amounts of MBL and ficolin-2. The negative result can therefore be due to the number of bacteria used during incubation. Also in our hands, pneumococci numbers below $1x10^9$ did not results in depletion of MBL or ficolin-2 in serum, whereas 1 $x10^{10}$ pneumococci depleted a significant amount of MBL from serum. Neth *et al*. also showed no binding of MBL to 10 serotypes of *S. pneumoniae*.[23] In this study flowcytometry was used, however, instead of using serum, they used purified MBL. The overall general methodology was comparable with our study and therefore the discrepancy is remarkable. Explanations might be the requirement of small molecular cofactors for MBL binding or that subsequently MASP-2 binding stabilises the complex, which is perhaps more likely. In a more recent study by Brouwer *et al*., direct binding of MBL on the surface of pneumococci was readily detectable by flowcytometry.

Deposition of MASP-2 on the bacterial cell surface can also be due to ficolin-2 binding. In this study we were able to detect binding of ficolin-2 to the pneumococci. Thus far binding of ficolin-2 on pneumococci has hardly been studied. Krarup *et al.* showed that ficolin-2 bound only to serotype 11 of a panel of 20 serotypes tested using the serum depletion assay.[24] Brouwer *et al.* found a weak binding of ficolin-2 to *S. pneumoniae* serotype 3 in a western blot of bacteria incubated with human serum.[25] Our panel of pneumococci included 2 serotype 11 strains of which one bound ficolin-2. As such, the binding intensity did not differ from other pneumococcal strains.

Our data suggest that ficolin-2 rather than MBL is mainly responsible for the deposition of MASP-2 on pneumococci. This conclusion is based on the finding that is a substantial number of patients MASP-2 binding can be detected in the absence of any MBL binding. Therefore, ficolin-2 can be an important mediator in the activation of lectin pathway of complement. For other microorganisms such as group B streptococci, it also has been demonstrated that MASP-2 binding can be mediated primarily through ficolin-2 binding.[26]

In some patients, binding of MASP-2 could not be demonstrated despite the apparent deposition of ficolin-2. This might be due to a MASP-2 polymorphism. Although we did not perform a genetic analysis of the MASP-2 gene in our cohort, this polymorphism is very rare in the normal population, as well as in patients with CAP, and therefore this explanation is unlikely.[27] Another explanation might be that the spatial configuration of ficolin-2 ligands on certain strains of pneumococci does not allow MASP-2 binding. Furthermore,

it is known that many microorganisms have developed methods to evade the innate immune system. Evasion from the lectin pathway of the complement system has not been studied in detail. However, it is possible that pneumococci are able to interfere with binding of ficolin-2 and subsequent binding of MASP-2. In this study, the MASP-2, MBL or ficolin-2 binding seems not to be correlated with pneumococcal serotype. When binding of these lectins would depend on specific sugars in the capsule polysaccharide of the pneumococcus, an association of serotype with binding would be expected. It is possible that binding of the lectins depends on other capsule constituents, like acidic components (like D-glucuronic acid or phosphate groups), ribitol, or arabinitol.[28] MASP-2, MBL and ficolin-2 binding may therefore depend on *S. pneumoniae* strain. Furthermore, it is generally assumed that the levels of expression of the pneumococcal capsule may vary during different phases. [29] All the variables may explain the fact that we did not find an association with serotype.

Two of the three patients homozygous deficient for the ficolin-2 +6359 SNP (associated with decreased binding capacity to GlucNAc) were able to bind ficolin-2 on pneumococci. In these two patients this homozygous SNP for decreased binding capacity was combined with a homozygous SNP for increased ficolin-2 levels (-4G/G). It is possible that ficolin-2 binds to other ligands on pneumococci or that the decreased binding is compensated by higher levels of ficolin-2. In the patient with homozygous deficient +6359 SNP T/T and normal -4A/A no ficolin-2 binding was seen. However, none of the three +6359 homozygous deficient patients were able to bind MASP-2. Due to a limited number of patients with a polymorphic genotype it is difficult to draw firm conclusions about genotype/phenotype relationship.

In conclusion, in this study we show that pneumococci can bind MASP-2. ficolin-2 appears to be the overriding MASP-2 activator. It is questionable whether pneumococcal serotype does influence MASP-2 binding. It is attractive to postulate that this route of the innate immune system contributes to host-defence against pneumococcal infections, but the functional consequences of ficolin-2 and MASP-2 binding for opsonophagocytosis and intracellular killing remained to be determined.

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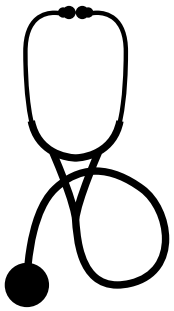

# Chapter 6

# Longitudinal analysis of pneumococcal antibodies during community-acquired pneumonia reveals a much higher involvement of *Streptococcus pneumoniae* than estimated by conventional methods alone

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## Abstract

### Background

In up to half of all cases of community-acquired pneumonia (CAP) no pathogen can be identified with conventional diagnostic methods. The most common identified causative agent is *Streptococcus pneumoniae*. In this study pneumococcal antibody responses during CAP were analysed to estimate the contribution of the pneumococcus to all cases of CAP for epidemiological purposes.

### Methods

Pneumococcal antibodies against 14 different serotypes were measured in serum of hospitalised CAP patients. Patients participated in one of two consecutive clinical trials in a general 600-bed teaching hospital in the Netherlands (between October 2004 and June 2009). A significant pneumococcal immune response was defined as at least a 2-fold increase in antibody concentrations against a single serotype between an early (day 1) and late (day 30) serum sample of each patient, with an end concentration above 0.35 µg/mL.

### Results

A total of 349 adult CAP patients participated in two consecutive clinical trials. For 200 patients, sufficient serum samples were available to determine antibody responses: 62 pneumococcal pneumonia patients, 57 nonpneumococcal pneumonia patients, and 81 patients with an unidentified causative agent. A significant immune response was detected in 45% (28/62 patients) of pneumococcal pneumonia patients, in 5% (3/57) of nonpneumococcal pneumonia patients and in 28% (23/81) of patients with an unidentified causative agent.

### Conclusion

The estimated contribution of pneumococci in patients with an unidentified causative agent was calculated to be 57% (95% confidence interval 36 – 86%). A substantial fraction of pneumococcal pneumonia patients does not elicit a serotype-specific immune response.

## Introduction

*Streptococcus pneumoniae*, the pneumococcus, is an important human pathogen causing serious diseases such as pneumonia, meningitis and sepsis in both children and adults.[1-5] The reported estimated mortality associated with invasive pneumococcal disease varies between 7 and 43%, depending on pneumococcal serotype, medical history of the patient and many other factors.[1, 6, 7] Community-acquired pneumonia (CAP) is one of the most common causes of death worldwide and the leading cause of death by infection in the United States.[8-10] *S. pneumoniae* is the most common identified pathogen in CAP.[9, 11, 12] It causes 13 to 48% of CAP cases requiring hospital admission. *S. pneumoniae* is also the most prevalent microorganism in mixed CAP.[9, 13] The exact contribution of the pneumococcus to all cases of CAP is not known as in 17 to 48% of CAP patients no definite causative agent can be identified.[9, 11, 12]

*S. pneumoniae* is surrounded by an external polysaccharide capsule. On basis of differences in the composition of this capsule, 92 pneumococcal serotypes have been identified. The capsular polysaccharide is the single most important trigger to the host immune response, which is serotype specific.[14] Anticapsular antibodies have been proven to be protective against pneumococcal infection. Thus, capsular polysaccharides form the basis of the available pneumococcal vaccines. A 23-valent polysaccharide vaccine is in use for adults.[3] For infants, a 7-valent conjugate vaccine has been widely introduced.[3, 5]

The response to vaccination is generally determined by measurement of antibody concentrations by serotype specific enzyme-linked immunosorbent assays (ELISA). With this method, monitoring antibody responses to at least the most prevalent pneumococcal serotypes requires high sample volumes and is time-consuming. Recently, a microsphere-based flow cytometric assay has been developed for simultaneous measurements of IgG antibody concentrations to 14 pneumococcal serotypes from a single sample (Luminex xMAP technology).[15] As opposed to vaccination response studies, few data exist on the immune response during pneumococcal infection.[16] The studies on anticapsular antibodies in pneumococcal pneumonia that have been performed have limited impact due to small patient groups, non-serotype specificity, lack of quantitative data, and/or outdated methodology.[14, 17-20]

In this study we measured serotype-specific antibody concentrations at different time points after the onset of CAP. Not only pneumococcal pneumonia patients but also patients infected with another respiratory pathogen or with an unidentified causative agent were included. By analysing antibody responses in these groups, we aimed to estimate the relative contribution of the pneumococcus to all cases of CAP.

## Methods

**Study population and clinical samples.**

Serum samples were obtained from patients above 18 years of age hospitalised with CAP who participated in two consecutive clinical trials.[21,22] Patients were hospitalised in the period October 2004 to August 2006 and November 2007 to June 2009 in a general 600-bed teaching hospital in the centre of the Netherlands. Inclusion and exclusion criteria are described in more detail elsewhere.[21, 22] CAP was defined as a new infiltrate on the chest X-ray, evaluated by an experienced radiologist, and at least 2 out of 6 clinical signs of pneumonia (cough, sputum production, temperature >38.0 °C or <36.0°C, abnormalities on auscultation compatible with pneumonia, leukocytosis, or leukopenia, C-reactive protein concentration of >15 mg/dL).[21, 22] Patients with a history of recent hospitalisation or a congenital or acquired immunodeficiency (including patients recently treated with prednisone 20 mg per day for more than 3 days in the first trial and all patients treated with corticosteroids in the second trial) were excluded. In the second clinical trial, patients were randomised to receive corticosteroids or placebo on admission. Serum samples were obtained at day 1 (day of admission), 2, 3, 5, 10 and 30. Sera were stored at –80 °C. Patient data collected were: age, sex, comorbid conditions (diabetes mellitus, chronic obstructive pulmonary disease, congestive heart failure, hepatic failure, renal failure and malignancies), duration of symptoms before hospital admission, use of antibiotics before admission, duration of hospital stay, and survival. The pneumonia severity index (PSI) was calculated on admission.[23]

**Pathogen identification**

The following diagnostic tests were performed on materials obtained at the day of admission to identify the causative agent of CAP. An expectorated sputum sample was Gram-stained and cultured, as were at least two blood samples (BacT/Alert, bioMérieux, Marcy l'Etoile, France). Sputum was considered representative if in the Gram-stained sample less than 25 epithelial cells per view (at x100 magnification) were present in the absence of leucocytes or if less than 50 epithelial cells per view were present in the presence of leucocytes. A respiratory pathogen cultured from sputum was only considered to be of aetiological significance if it was cultured in relative abundance to the commensal flora of the throat and if the Gram-stain revealed the microorganism in abundance (>10 microorganisms per view at x1,000 magnification). Taqman real-time PCRs were performed with sputum in order to detect DNA of atypical pathogens (*Mycoplasma pneumoniae*, *Legionella pneumophila*, *Coxiella burnetii* and *Chlamydophila pneumoniae*, and C. *psittaci*). Urine samples were used for detection of *S. pneumoniae* and *L. pneumophila* serogroup 1 antigens

(BinaxNOW *S. pneumoniae* and BinaxNOW *Legionella*; Inverness Medical). Paired serum samples (the second sample obtained 14 to 21 days after admission) were tested for the development of antibodies to *M. pneumoniae*, *C. burnetii*, *L. pneumophila*, and respiratory viruses by complement fixation reactions or standard ELISA. Pharyngeal swab samples were taken for viral culture and viral PCR. The diagnostic protocol is described in more detail elsewhere.[22] On the basis of the causative agent identified, patients were divided into three groups: pneumococcal CAP, nonpneumococcal CAP and CAP with an unidentified causative agent. *S. pneumoniae*, cultured from either sputum or blood, was serotyped by the Quellung reaction.

**Measurement of pneumococcal polysaccharide antibody concentrations**

Antibodies were measured in two serum samples of the CAP patients: an early sample drawn at day 1 (day 0 to 3) after hospital admission and a late sample drawn at day 30 (days 11 to 100). Patients with a duration of symptoms of more than 10 days before hospital admission were excluded, as well as patients for whom the interval between the earliest and latest serum sample available was less than 10 days. Antibodies against pneumococcal polysaccharides were measured on a Luminex platform (Luminex Corporation, Austin, TX), using a quantitative multiplex immunoassay (MIA): the XMAP pneumococcal immunity panel. This assay identifies serotype-specific anti-capsular polysaccharide IgG antibodies to the following serotypes: 1, 3, 4, 8, 9, 12, 14, 19, 23, 26, 51, 56, 57, and 68 according to the American nomenclature, corresponding to serotypes 1, 3, 4, 8, 9N, 12F, 14, 19F, 23F, 6B, 7F, 18C, 19A, and 9V, respectively according to the Danish nomenclature. Samples were diluted by a factor of 1:100 with diluent solution, included in the XMAP kit, composed of phosphate buffered saline (PBS), pH 7.3, with pneumococcal cell wall polysaccharide (C-PS) and polysaccharide 22 (PS-22) added at working concentrations to inhibit nonspecific binding of anti-cell wall polysaccharide I and II. Diluted sera were incubated with a mixture of 14 microsphere types, each coated with antigen representing 1 of 14 pneumococcal serotypes. Nonbound antibodies were washed away and each sample was treated with phycoerythrin-conjugated goat anti-human IgG. Unbound conjugate was washed away, and the bead suspensions were analysed on the Luminex analyser (IS 2.3). Seven standard dilutions, included in the XMAP kit, calibrated to FDA 89-SF reference serum, were used to generate a standard curve for quantification of antibody concentrations. Three assay controls were prepared in duplicate with the test samples in each assay.

### Statistical analyses

Microsoft Excel software (version 2000) and SPSS software (version 17.0) were used for statistical analyses. A significant increase in antibody concentrations, or a positive immune response, was defined as at least a 2-fold increase between the early and late serum sample of each patient with an end concentration above 0.35 µg/mL. The fold increase in antibody concentrations against a single pneumococcal serotype had to be at least two times greater than the fold increase against any other serotype. It was first assumed that all patients infected with *S. pneumoniae* have the same probability of a positive pneumococcal immune response. A second assumption was that positive pneumococcal immune responses detected in patients in whom another pathogen was identified by conventional methods, were falsely positive. The probability of mixed infections was neglected. On the basis of these assumptions, the contribution of the pneumococcus in CAP patients with an unidentified causative agent was estimated by the positive predictive value (PPV), corrected by the sensitivity of the test. The PPV of a significant increase was calculated from results in all definite pneumococcal and definite nonpneumococcal pneumonia patients. The sensitivity of the test was established from results in pneumococcal pneumonia patients. Groups were compared by using the Student's t test and Fisher's exact test where appropriate. A p-value of $<0.05$ was considered to represent a statistically significant difference.

## Results

### Pathogens

A total of 349 CAP patients participated in two clinical trials (clinical trial 1, 200 patients; clinical trial 2, 149 patients). In 94 patients (27%), *S. pneumoniae* was diagnosed as the causative agent, in 86 patients (25%) a pathogen other than *S. pneumoniae*, and in 169 patients (48%) no pathogen was identified (*Figure 1*). Demographic data and disease characteristics of the patients are shown in *table 1*. The most commonly identified pathogens in the nonpneumococcal pneumonia patients were *C. burnetii* (n = 19), *Legionella* species (n = 15), *M. pneumoniae* (n = 11), *Haemophilus influenzae* (n = 7), C. *psittaci* (n = 7), influenza A virus (n = 6) and *Staphylococcus aureus* (n = 4). In 29 (31%) of the pneumococcal pneumonia patients, diagnosis was based on a positive blood culture with *S. pneumoniae*, in 40 patients (43%) blood cultures remained negative but the urine antigen test for *S. pneumoniae* was positive and in 25 patients (27%) aetiologic diagnosis was solely based on a positive sputum culture with a Gram-stain revealing purulence and Gram positive diplococci in abundance. Pneumococcal strains isolated from 42 patients were available for serotyping (29 blood isolates, 13 sputum isolates). The most prevalent serogroups were serogroup 1 (n = 8),

9 (n = 8, of which serotype 9V n = 3, serotype not defined n = 5), 14 (n = 5), 8 (n = 4) and 3 (n = 4). Twenty-nine isolates (69%) had a serotype covered by the 14-plex panel used to measure antibodies, 7 isolates had a serogroup covered by the panel but could not be further subtyped due to technical reasons (serogroup 7 and 9) and 6 isolates were of a serotype not covered by the panel.

**Table 1.** Demographic data and disease characteristics of 349 community-acquired pneumonia patients grouped by causative agent

| Characteristics | *Streptococcus pneumoniae* (n = 94) | Different pathogen (n = 86) | Unidentified pathogen (n = 169) | Total (n = 349) |
|---|---|---|---|---|
| **Age, years** | 60 | 56 | 68[a] | 63 |
| **Sex, male[b]** | 48 (51) | 58 (67)[c] | 101 (60) | 207 (59) |
| **Patients with one or more comorbid conditions[d]** | 44 (47) | 32 (37) | 80 (47) | 156 (45) |
| **PSI[e] on admission** | 87 | 81[f] | 93 | 88 |
| **Patients with antibiotic use before admission** | 13 (14)[g] | 30 (35) | 47 (28) | 90 (26) |
| **Duration of hospitalisation (days)** | 14 | 11 | 12 | 12 |
| **Patients who survived** | 90 (96) | 81 (94) | 160 (95) | 331 (95) |

Data are presented as mean or number (%)
[a]: Significant difference ($p<0.05$) by Student's t test, compared to other two pathogen groups.
[b]: Percentages are calculated as the number of patients within the pathogen group
[c]: Significant difference ($p<0.05$) by Fisher's exact test, compared to *S. pneumoniae* group.
[d]: Co-morbid condition is one of more of the following: diabetes mellitus, chronic obstructive pulmonary disease, congestive heart failure, hepatic failure, renal failure and malignancies.
[e]: PSI is described elsewhere[10]
[f]: Significant difference ($p<0.05$) by Student's t test compared to unidentified pathogen group.
[g]: Significant difference ($p<0.05$) by Fisher's exact test, compared to other two pathogen groups.

### Pneumococcal polysaccharide antibody concentrations

Of 200 patients, sufficient serum samples were available to determine antibody response (110 from clinical trial 1 and 90 from clinical trial 2). All 200 patients were included in the study: 62 pneumococcal pneumonia patients, of whom 20 patients with an identified serotype of the infecting strain covered by the 14-plex antibody pane; 57 nonpneumococcal pneumonia patients; and 81 patients with an unidentified causative agent (*Figure 1*). No differences in immune response rates were found between patients of the two trials or between patients who did and did not receive corticosteroids in trial 2.

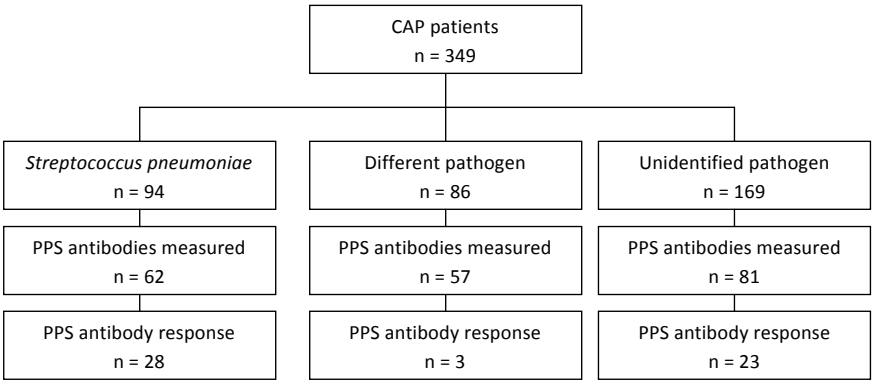


**Figure 1.** Flowchart of study design. A total of 349 CAP patients participated in two clinical trials. In 94 patients (27%) *Streptococcus pneumoniae* was found to be the causative agent by conventional microbiological techniques, in 86 patients (25%) a pathogen other than *S. pneumoniae* was identified, and in 169 patients (48%) no pathogen was identified. Sufficient samples for pneumococcal antibody measurements were available for 200 patients. A pneumococcal polysaccharide (PPS) antibody response was defined as at least a 2-fold increase in antibody concentrations with an end concentration above 0.35 g/ml. The fold increase against a single pneumococcal serotype had to be at least two times greater than the fold increase against any other serotype.

**Antibody responses in pneumococcal CAP**

Twenty-eight (45%) of 62 pneumococcal pneumonia patients elicited a significant increase in antibody concentrations against a single pneumococcal serotype (*Table 2*). Of the 18 patients with a positive blood culture, 8 (44%) elicited an immune response whereas 20 (45%) of 44 patients in whom the aetiologic diagnosis was based on a positive sputum culture and/or a positive urine antigen test elicited an immune response. In patients with a positive immune response, mean PSI at admission was lower and duration of hospital stay was shorter than those patients that failed to elicit an immune response. Of the 20 pneumococcal pneumonia patients infected with an identified *S. pneumoniae* serotype covered by the 14-plex antibody panel, 11 (55%) elicited a significant immune response, all against the infecting serotype (*Table 2*). Of the 14 patients in whom the strain was isolated from blood, 6 (43%) elicited a response, whereas 5 (83%) of the 6 patients in whom the strain was isolated from sputum elicited a response. End concentrations varied from 0.74 to 12.24 µg IgG/mL (*Table 3*). Five of the nine patients that failed to elicit an immune response were infected with *S. pneumoniae* serotype 1. Of the patients infected with a pneumococcal serotype not included in the 14-plex panel, none elicited a significant antibody response.

**Table 2.** Clinical characteristics of pneumococcal pneumonia patients grouped by immune response

| Group and Characteristics | Positive immune response[a] | Negative immune response[a] | Total |
|---|---|---|---|
| **All pneumococcal pneumonia patients** | | | |
| Patients in group | 28 | 34 | 62 |
| Age, years | 56 | 61 | 59 |
| Sex, male[b] | 14 (50) | 17 (50) | 31 (50) |
| Patients with one or more comorbid conditions[c] | 11 (39) | 19 (56) | 30 (48) |
| PSI[d] on admission | 74[e] | 91 | 83 |
| Patients with antibiotic use before admission | 6 (21) | 2 (6) | 8 (13) |
| Patients with aetiologic diagnosis by: | | | |
| Blood Culture | 8 (29) | 10 (29) | 18 (29) |
| Urine antigen test | 11 (39) | 17 (50) | 28 (45) |
| Sputum culture | 9 (32) | 7 (21) | 16 (26) |
| Duration of hospitalisation (days) | 10[e] | 15 | 13 |
| Patients who survived | 28 (100) | 34 (100) | 62 (100) |
| **Pneumococcal pneumonia patients with identified serotype of infecting strain within antibody-panel** | | | |
| Patients in group | 11 | 9 | 20 |
| Age, years | 50 | 55 | 52 |
| Sex, male | 5 (45) | 8 (89) | 13 (65) |
| Patients with one or more comorbid conditions | 4 (36) | 2 (22) | 6 (30) |
| PSI on admission | 69 | 96 | 81 |
| Patients with antibiotic use before admission | 1 (9) | 0 (0) | 1 (5) |
| Patients with the following source of isolate: | | | |
| Blood culture | 6 (55) | 8(89) | 14 (70) |
| Sputum culture | 5 (45) | 1 (11) | 6 (30) |
| Duration of hospitalisation (days) | 9 | 16 | 12 |
| Patients who survived | 11 (100) | 9 (100) | 20 (100) |

Data are presented as mean or number (%)
[a]: Response was defined as at least 2-fold increase in antibody concentrations with an end concentration above 0.35 µg/ml. The fold increase against a single pneumococcal serotype had to be at least two times greater than the fold increase against any other serotype.
[b]: Percentages are calculated from the number of patients within the response group.
[c]: Comorbid condition is one or more of the following: diabetes mellitus, chronic obstructive pulmonary disease, congestive heart failure, hepatic failure, renal failure and malignancies.
[d]: PSI is described elsewhere[10]
[e]: Significant differences ($p<0.05$) by Students t-test compared to negative immune response group

**Table 3.** Antibody concentrations against the infecting serotype in 20 patients infected with *Streptococcus pneumoniae*

| Serotype of infecting strain | Positive immune response[a] (n = 11) | | | Negative immune response[a] (n = 9) | | |
|---|---|---|---|---|---|---|
| | Concentration (µg/ml) | | Fold increase | Concentration (µg/ml) | | Fold increase |
| | Early sample[b] | Late sample[c] | | Early sample | Late sample | |
| **1** | 0.54 | 3.82 | 7.12 | 0.37 | 0.57 | 1.54[d] |
| | | | | 0.14 | 0.31 | 2.18[d] |
| | | | | 0.32 | 0.30 | 0.95[d] |
| | | | | 2.30 | 4.34 | 1.88[d] |
| | | | | 0.02 | 1.09 | 43.94[d] |
| **3** | 0.06 | 1.38 | 24.08[d] | | | |
| | 0.10 | 1.71 | 16.45 | | | |
| **4** | 0.01 | 1.47 | 100.95[d] | 0.10 | 0.14 | 1.41[d] |
| **8** | 0.26 | 0.74 | 2.86[d] | 0.09 | 0.10 | 1.21 |
| | 1.22 | 12.24 | 10.04 | | | |
| **9V** | 0.20 | 1.42 | 7.16 | 0.10 | 0.13 | 1.28[d] |
| | | | | 0.09 | 0.22 | 2.53[d] |
| **14** | 0.02 | 1.67 | 110.89[d] | | | |
| | 0.05 | 1.15 | 22.84[d] | | | |
| | 0.05 | 3.35 | 66.74 | | | |
| **19F** | 0.11 | 6.03 | 54.76[d] | | | |

[a]: Response was defined as at least a 2-fold increase in antibody concentrations, with an end concentration above 0.35 µg/ml. The fold increase against a single pneumococcal serotype had to be at least two times greater than the fold increase against any other serotype. All positive antibody responses were against the infecting serotype.
[b]: The early serum sample was drawn at day 1 (days 0 to 3) of hospital admission.
[c]: The late serum sample was drawn at day 30 (days 11 to 100) of hospital admission.
[d]: Strain was isolated from blood.

### Antibody responses in nonpneumococcal CAP

Of the 57 CAP patients in whom a pathogen other than *S. pneumoniae* was found, 3 (5%) elicited a pneumococcal antibody response. In 2 of the 3 patients, *L. pneumophila* was identified by a positive PCR. The third patient was infected with C. *psittaci*, identified by a positive PCR and the development of antibodies in paired serological testing.

### Antibody responses in CAP with unidentified causative agent

Of the 81 CAP patients with an unidentified causative agent, 23 patients (28%) elicited an increase in antibody concentrations against a single pneumococcal serotype. The PPV of a significant immune response, calculated from

antibody responses in definite pneumococcal and definite nonpneumococcal pneumonia patients, was 28/(28+3) = 0.90 (95% confidence interval [CI] 0.74 – 0.98). Calculated sensitivity was 28/62 = 0.45 (95% CI 0.32 – 0.58). The estimated contribution of the pneumococcus in the 81 patients with an unidentified causative agent was calculated as follows: number of patients in this group with a positive immune response x positive predictive value x inverse sensitivity = 23 x 0.90 x (1/0.45) = 46 patients (57%, 95% CI 36 – 86%).

### Serotype distribution of antibody responses

*Table 4* shows the serotype distribution of significant antibody responses. A response against serotype 14 was most prevalent, followed by responses against serotype 3, 7F and 8. The serotype distribution of antibody responses in pneumococcal CAP patients was similar to that in CAP patients with an unidentified cause.



**Table 4.** Serotype distribution of significant pneumococcal antibody responses in community-acquired pneumonia patients grouped by causative agent

| Serotype immune response | No. of patients | | | |
|---|---|---|---|---|
| | ***Streptococcus pneumoniae*** **(n = 28)** | **Different pathogen (n = 3)** | **Unidentified pathogen (n = 23)** | **Total (n = 54)** |
| **14** | 5 | 0 | 4 | 9 |
| **3** | 2 | 1 | 6 | 9 |
| **7F** | 7 | 0 | 1 | 8 |
| **8** | 4 | 0 | 4 | 8 |
| **9V** | 2 | 1 | 3 | 6 |
| **4** | 2 | 0 | 3 | 5 |
| **1** | 2 | 1 | 0 | 3 |
| **23F** | 1 | 0 | 1 | 2 |
| **9N** | 1 | 0 | 0 | 1 |
| **18C** | 1 | 0 | 0 | 1 |
| **19F** | 1 | 0 | 0 | 1 |
| **6B** | 0 | 0 | 1 | 1 |

## Discussion

Identification of the causative agent of CAP is notoriously difficult. Because *S. pneumoniae* appears to be the most common pathogen causing CAP, many new diagnostic methods are being developed for the detection of this microorganism. The diagnostic yield of these, mostly PCR-based, methods is

generally low.[11, 24, 25] In this serology-based study, the estimated contribution of the pneumococcus in patients with an unidentified causative agent was 57%, a strikingly high proportion. This results in an estimated involvement of *S. pneumoniae* in 190 of all 349 (54%) CAP cases in our study: 94 patients identified by conventional methods and 96 patients estimated by a positive immune response (0.57 x 169 patients). These results are of primary importance for epidemiological reasons. As a late serum sample has to be obtained to monitor an antibody response, this serological diagnostic method is less useful for diagnosis of pneumococcal infection in the acutely ill individual patient.

The distribution of pathogens identified by conventional methods was similar to that found in earlier studies.[9] The high incidence of *S. pneumoniae* serotype 1 was striking.[6] Introduction of the 7-valent conjugate pneumococcal vaccine, which does not include serotype 1, for children in the Netherlands in 2006 may have caused the increased incidence of this serotype. A large national epidemiological study will be conducted on this subject. The high incidence of *C. burnetii* was due to large outbreaks of Q fever in the Netherlands from 2007 to 2009.[26]

Forty-five percent of patients in whom *S. pneumoniae* was found to be the causative agent elicited an immune response (*Figure 1*; *Table 2*). Only immune responses against a limited number of serotypes could be detected, but also, in the patients known to be infected with a serotype included in the 14-plex antibody panel, the response rate was only 55% (*Table 2*; *Table 3*). Failure to elicit an antibody response could be the primary risk factor for CAP. In a follow-up study, CAP patients therefore will receive pneumococcal vaccination to monitor their ability to elicit a serotype-specific antibody response. The response rate after pneumococcal polysaccharide vaccination varies from 25 to 100% in a healthy population, depending on serotype.[16]

The high mean PSI at admission and the long duration of hospital stay in pneumococcal pneumonia patients that failed to elicit an immune response compared to those for patients who did elicit a response are suggestive of more severe disease in the nonresponding patients (*Table 2*). Age and medical history of the patients are expected to be the most important factors contributing to these differences, but in our study, differences in age and comorbid conditions were not significant between responders and nonresponders. In earlier studies, the immune response after pneumococcal vaccination was diminished in the elderly, as was the occurrence of naturally acquired antibodies.[27, 28] There was a nonsignificant difference in antibiotic use before admission between the two response groups. It could be speculated that antigen presentation was more effective in patients in whom (part of) the bacteria had been killed by antibiotics, resulting in a positive immune response. Within the group of patients with

an identified serotype of the infecting strain included in the antibody panel, in most nonresponders *S. pneumoniae* was cultured from blood, although differences were not significant (*Table 2*). Failure to elicit an antibody response could have facilitated bacteremia in these patients. Most pneumococcal pneumonia patients who did not elicit an immune response were infected with *S. pneumoniae* serotype 1 (*Table 3*). In earlier studies immunogenicity of this serotype was intermediate.[16, 29]

For the calculation of the contribution of *S. pneumoniae* in CAP, the assumption was made that the probability of a positive pneumococcal immune response in patients infected with *S. pneumoniae* that was not detected by conventional methods was equal to the probability in patients in whom the pneumococcus was detected by conventional methods. This is a conservative approach, as it is reasonable to assume that the sensitivity of the pneumococcal antibody measurements was lower in patients in whom no causative agent could be identified. Diagnostic tests could have remained negative in these patients due to a low burden of pathogens that was below the threshold needed to trigger an immune response. Furthermore, the mean age of patients with an unidentified causative agent was significantly higher, and older age is also a well-known factor associated with impaired responses to pneumococcal polysaccharides (*Table 1*).[27, 28]

The second assumption made for statistical calculations regards the probability of co-infections. Of 57 patients in whom a pathogen other than *S. pneumoniae* was established to be the causative agent, 3 patients (5%) elicited a pneumococcal immune response (*Figure 1*). CAP caused by multiple infecting microorganisms has been described to occur at incidences varying from 2 to 13 %.[9,13] *S. pneumoniae* is the most prevalent microorganism in mixed CAP. A pneumococcal immune response in a nonpneumococcal pneumonia patient could be regarded as a sign of double infection. Indeed, a potential application of the measurement of pneumococcal antibodies during CAP could be to improve the otherwise difficult identification of *S. pneumoniae* in mixed infections. Nevertheless, for conservative calculations in this study, immune responses in nonpneumococcal pneumonia patients were considered as false positives.

In this study, as in studies on the antibody response to vaccination, absolute values of antibody concentrations did vary considerably both between different pneumococcal serotypes and between patients infected with the same serotype (*Table 3*).[16] This variation complicates the application of a strict definition of a positive immune response. In this study a significant antibody response was defined as at least a 2-fold increase in antibody concentration at least 2-fold greater than the fold increases against the other serotypes with

an end concentration above 0.35 µg/mL. This definition was derived from previous studies on the antibody response to pneumococcal vaccination.[16, 27] The threshold of 0.35 µg/mL is the designated protective concentration after pneumococcal conjugate vaccination in children recommended by a WHO Working Group.[30] For the purpose of this study, 0.35 µg/mL is an arbitrary threshold and does not have any functional meaning. The 2-fold-increase cut-off was also defined rather arbitrarily. *Post-hoc* analysis revealed that using instead a threshold of 0.17 or 0.70 µg/ or a 3- or 4-fold increase as cut-off for the definition of a positive immune response, response rates were equivalent and resulted in similar estimations of the contribution of the pneumococcus in all cases of CAP. Compared to 28, 3 and 23 positive immune responses in the pneumococcal, nonpneumococcal and unidentified pathogen groups, respectively, using the applied definition, the respective numbers would be 29, 3 and 22 using a 0.17 µg/mL threshold; 27, 3, and 22 using a 0.70 µg/mL threshold; 27, 2 and 21 using a 3-fold increase cut-off and 27, 2 and 20 using a 4-fold increase cut-off. These results justify the definition of a positive immune response used in this study.

Measurement of pneumococcal anticapsular antibodies was shown to be a useful diagnostic tool in serotype-specific identification of *S. pneumoniae* as the causative agent of CAP. Twenty-eight percent of CAP patients in whom no causative agent could be identified with conventional diagnostic tests elicited a serotype-specific pneumococcal immune response. Taking into account the positive predictive value and sensitivity of the test, a total of 57% (95% CI = 36 to 86%) of these patients were estimated to have been infected with *S. pneumoniae*. A surprisingly large fraction of CAP patients infected with *S. pneumoniae* did not elicit a serotype-specific immune response.

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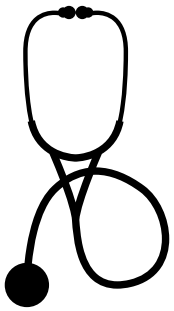
# Part 3

## Biomarkers



# Chapter 7

# Prognostic value of serum angiotensin-converting enzyme activity for outcome of community-acquired pneumonia

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## Abstract

### Background

In a previous study, a relation between decreased serum angiotensin-converting enzyme (ACE) activity and physiological parameters was observed in patients with community-acquired pneumonia. The present study aims to further assess the prognostic value of serum ACE activity for outcome of community-acquired pneumonia.

### Methods

This was a prospective observational study including two cohorts of patients with community-acquired pneumonia (2004–2006; n=157 and 2007–2010; n=138). Serum ACE activity was measured at time of hospital admission. Based on reference values in healthy persons, patients were divided into subgroups of serum ACE activity: normal, low and extremely low. Physiological parameters, clinical outcomes and aetiology were compared between the subgroups.

### Results

A total of 265 patients were enrolled in this study. Mean age was 60±19 years. In patients with low serum ACE activity (< 20U/L, n=53), compared to patients with normal serum ACE activity (> =20 U/L, n=212), C-reactive protein (CRP) was significantly increased, systolic blood pressure was significantly lower and there was a trend for higher heart rate and leukocyte counts. Furthermore, *Streptococcus pneumoniae* was significantly more identified in patients with low serum ACE activity. Serum ACE activity <24 U/L was independently associated with bacteremia (adjusted OR 3.93 [95% CI 1.57 – 9.87]). Low serum ACE activity was not prognostic for length of hospital stay nor mortality.

### Conclusion

This study did not show prognostic value for serum ACE activity regarding clinical outcome in patients with community-acquired pneumonia. Serum ACE activity <24 U/L at time of hospitalisation appeared an independent indicator for the presence of bacteremia. Further research should elucidate the role of ACE in systemic infection and sepsis during pneumonia.

## Introduction

Community-acquired pneumonia (CAP) is the leading community-acquired infection needing hospitalisation, with mortality rates up to 20% for hospitalised patients.[1-3] The high incidence in combination with wide variation in clinical course stresses the need for effective assessment tools to distinguish between patients at high risk for clinical deterioration and patients suitable for early hospital discharge or home management. Several biomarkers and risk scores, such as the pneumonia severity index (PSI)[4] are being used by clinicians to manage patients presenting with symptoms of pneumonia. So far, most available markers lack specificity. To better identify patients at risk for adverse outcome other possible biomarkers need to be explored. Serum angiotensin I – converting enzyme (ACE) activity has been found to decrease during pneumonia and to return to normal values during recovery.[5,6] The clinical significance and biological explanation of this temporary decrease remain unclear, but it may play a role in the homeostasis during pneumonia.

ACE is well known for its role in blood pressure and electrolyte homeostasis. In the renin-angiotensin-aldosterone system (RAAS), ACE cleaves angiotensin I to generate angiotensin II, a powerful vasoconstrictor and stimulator of aldosteron secretion.[7] ACE has a wide tissue and cellular distribution, and is mostly expressed on the luminal membrane of vascular endothelial cells, in particular of the pulmonary endothelium. After cleavage, endothelial ACE is released into the circulation. Serum ACE originates mainly from lung micro-vessels[8-10] and it has been studied in various pulmonary disorders. Besides in pneumonia, serum ACE activity is also decreased in adult respiratory distress syndrome (ARDS) and in sepsis.[11-15]

In a previous study a negative correlation between serum ACE activity and a combination of physiological parameters was observed[6], as well as a trend towards worse outcome in patients with low serum ACE activity at the time of hospital admission. Due to low sample size, this study was not able to test the association with outcome of CAP satisfactorily. Therefore, in the current analysis, a second cohort is adjoined to the first cohort. This prospective observational study aims to further assess the usefulness of serum ACE activity at the time of hospital admission as a prognostic marker.

## Methods

### Study design and patients

This study is conducted in continuation of a previous study. In the present study, a second cohort of patients is added to the study cohort described previously.[5] Both cohorts included patients with confirmed pneumonia, admitted to

the St. Antonius Hospital in Nieuwegein, the Netherlands. The first cohort consists of patients admitted between October 1, 2004 and 1 August 1, 2006 and is described elsewhere. The second cohort consists of patients admitted with CAP between November 1, 2007 and April 1, 2010. Inclusion criteria, pathogen identification and ACE measurement were identical for both cohorts. Pneumonia was defined as a new infiltrate on a chest radiograph plus at least two of the following criteria: cough; sputum production; temperature >38°C or <35.5°C; auscultatory findings consistent with pneumonia, leukocytosis, or leukopenia (>10 G/L, <4 G/L, or 10% rods in leukocyte differentiation); and C-reactive protein (CRP) greater than three times the upper limit of normal. Patients who were immune compromised (haematologic malignancies, and other immunosuppressive therapy including systemic corticosteroids) and patients using ACE inhibitors or angiotensin II receptor blockers (ARBs) in the month before hospital admission were excluded. In both cohorts the same treatment guidelines concerning the use of antibiotics for pneumonia were used. Half of the patients from the second cohort received dexamethasone 5mg once daily for 4 days because of studies purposes. Dexamethasone was administered after blood was drawn for culture and ACE activity testing. The study was approved by the local medical Ethics Committee and all patients gave written informed consent. The ethnicity of the population in and around the city of Nieuwegein is primarily (94%) white Caucasian.

**Serum ACE activity and classification**

Blood samples were collected into lithium heparin tubes at the time of hospitalisation (day 1) and before the start of treatment. ACE activity was quantified using the Bühlmann ACE kinetic test, according to previously described methods (Bühlmann Laboratories AG, Switzerland).[16,17] The manufacturers' analytical sensitivity is <5 U/L. Based on a mean serum ACE activity of 39.7 U/L (SD 16.0, coefficient of variation 40.3%) in healthy volunteers[18], the following serum ACE activity cut-off points were composed. Serum ACE activity <20 U/L was defined as low serum ACE activity, corresponding with lowest 10% of the healthy reference group (mean serum ACE activity –1.28 x SD, i.e. Z-score ≤–1.28). Furthermore, serum ACE activity <14 U/L was defined as extremely low serum ACE activity, corresponding to the lowest 5% of the healthy reference group (mean serum ACE activity –1.64 x SD, i.e. Z-score ≤–1.64). Serum ACE activity between 14 and 19 U/L was defined as moderately low serum ACE activity. Serum ACE activity >=20 U/L was defined as normal serum ACE activity.

**Outcome measures and illness severity assessment**

The occurrence of the following clinical outcomes has been assessed for all patients: time to hospital discharge (without need for intensive care unit (ICU) admittance or patient death), need for ICU admittance and in hospital mortality. Patients in this study who were admitted to the ICU were in need for mechanical ventilation, vasopressor support or both.

In addition, the pneumonia severity index (PSI) was calculated for all patients.[19]

**Pathogen identification**

The diagnostic tools used to identify the causative microorganism of CAP have been described before.[20] In short, at least two sets of separate blood, and sputum samples from each patient were cultured. Sputum was analysed by in-house developed polymerase chain reaction (PCR) for atypical pathogens (*Mycoplasma pneumoniae*, *Legionella pneumophila*, *Coxiella burnetii* and *Chlamydophyla pneumoniae* and *Chlamydophyla psittaci*). Urine was sampled for antigen testing on *Streptococcus pneumoniae* and *L. pneumophila* serogroup 1. In addition, serum samples of the day of hospital admission and day 10 were analysed in pairs for detection of a fourfold rise of antibodies to respiratory viruses, *S. pneumoniae*[21], *M. pneumoniae*, *C. burnetii* and *C. psittaci* by complement fixation assay. Pharyngeal samples were taken for viral culture. Bacteremia was defined as at least one positive blood culture with the causative organism.

**Statistical analyses**

Data were analysed using SPSS (version 16.0; SPSS Inc., Chicago, IL, USA). Descriptive statistics of demographic and clinical variables were expressed as frequencies (percentages (%)) or mean (standard deviation (SD)) where appropriate. Categorical data were analysed by Chi-square or Fisher's Exact tests and continuous data by Students' t-tests or Mann-Whitney U-tests. In case of significant differences in outcome frequencies between normal and low ACE activity subgroups, receiver operating characteristic (ROC) curve analyses for serum ACE activity were performed to identify cut-off points with optimal predictive value. For that outcome, the same was conducted for other associated clinical variables ($p<0.10$). Using these cut-off points (dichotomization), a backward stepwise conditional logistic regression analysis was performed in order to assess the prognostic value of serum ACE activity for that outcome, independently from other possible predictors. A two-tailed p-value $<0.05$ was considered significant. *Post-hoc*, it was calculated that the present study had sufficient statistical power (alpha=0.05, power=0.80) to prove a 2.3-fold difference in mortality rate between patients with low and normal ACE activity (when observed).

## Results

### Patient characteristics

A flow chart of the patient stratification is shown in *figure 1*. Patient characteristics are shown in *table 1*. Demographics and clinical characteristics did not differ between the two cohorts. From this point on, all analyses are conducted in the total of 265 patients (i.e., the sum of both cohorts). Overall, mean serum ACE activity was 30.0 U/L (SD 14.9, coefficient of variation 49.7%). In patients with low serum ACE activity, systolic blood pressure was lower, CRP levels and leukocyte counts were higher ($p<0.05$, $p<0.01$ and $p<0.01$, respectively). The increase in CRP levels was even more evident in patients with extremely low serum ACE activity ($p<0.01$).

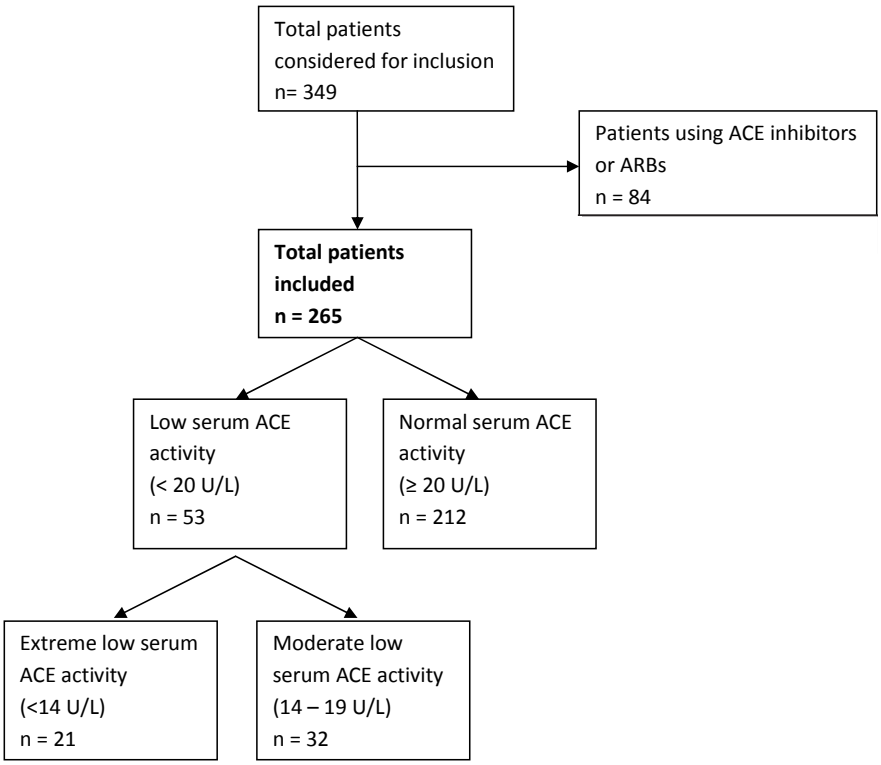


**Figure 1.** Flow chart of the patient stratification.

**Table 1.** Baseline characteristics of patients in the two study cohorts and in the subgroups of serum ACE activity

| | | | | | | | | Subdivision of low serum ACE activity group | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **All patients** | **Cohort 1** | **Cohort 2** | **p-value** | **Low ACE < 20 U/L** | **Normal ACE >= 20 U/L** | **p-value[a]** | **Extremely low ACE < 14 U/L** | **Moderately low ACE 14 – 19 U/L** | **p-value[b]** |
| Subjects, n[c] | 265 | 125 | 140 | | 53 | 212 | | 21 | 32 | |
| **Demographics** | | | | | | | | | | |
| Sex, male | 146 (55) | 75 (60) | 71 (51) | NS | 28 (53) | 118 (56) | NS | 10 (48) | 18 (56) | NS |
| Age, years | 60 (19) | 60 (20) | 59 (20) | NS | 58 (18) | 60 (19) | NS | 57 (19) | 60 (18) | NS |
| Comorbidities | | | | | | | | | | |
| COPD | 53 (20) | 38 (30) | 15 (11) | <0.01 | 8 (15) | 45 (21) | NS | 1 (4.8) | 7 (22) | NS |
| Chronic heart failure | 23 (8.7) | 9 (7.2) | 14 (10) | NS | 3 (5.7) | 20 (9.4) | NS | 2 (9.5) | 1 (3.1) | NS |
| Renal disease | 9 (3.4) | 3 (2.1) | 6 (4.8) | NS | 2 (3.8) | 7 (3.3) | NS | 0 (0.0) | 2 (6.3) | NS |
| Malignancy | 23 (8.7) | 11 (8.8) | 12 (8.6) | NS | 3 (5.7) | 20 (9.4) | NS | 1 (4.8) | 2 (6.3) | NS |
| Diabetes mellitus | 29 (11) | 14 (11) | 15 (11) | NS | 5 (9.4) | 24 (11) | NS | 3 (14) | 2 (6.3) | NS |
| **Physiology on admission** | | | | | | | | | | |
| Systolic BP (mmHg) | 132 (22) | 134 (26) | 131 (21) | NS | 124 (21) | 134 (23) | <0.05 | 126 (18) | 125 (22) | NS |
| Diastolic BP (mmHg) | 75 (13) | 75 (16) | 75 (12) | NS | 72 (14) | 75 (13) | NS | 72 (11) | 73 (15) | NS |
| Heart rate (beats/minute) | 98 (20) | 102 (22) | 97 (21) | NS | 102 (23) | 97 (21) | NS | 106 (21) | 97 (22) | NS |
| RR (breaths/minute) | 23 (7) | 23 (8) | 23 (6) | NS | 23 (7) | 23 (7) | NS | 23 (6) | 23 (8) | NS |
| Temperature | 38.2 (1.2) | 38.2 (1.1) | 38.2 (1.1) | NS | 38.1 (1.1) | 38.2 (1.2) | NS | 38.5 (1.1) | 37.9 (1.0) | NS |
| Hypothermia (n=22) | 21 (7.8) | 8 (6.5) | 13 (9.3) | NS | 3 (5.7) | 18 (8.5) | NS | 0 (0.0) | 3 (9.4) | NS |
| Hyperthermia (n=121) | 59 (41) | 49 (40) | 59 (42) | NS | 20 (38) | 88 (42) | NS | 10 (48) | 10 (31) | NS |
| **Clinical score** | | | | | | | | | | |
| PSI | 84 (39) | 83 (36) | 85 (41) | NS | 84 (40) | 85 (40) | NS | 86 (43) | 85 (38) | NS |
| **Infection markers** | | | | | | | | | | |
| CRP (mg/L) | 221 (142) | 201(153) | 233 (137) | NS | 294 (146) | 204 (136) | <0.01 | 377 (144) | 245 (144) | <0.01 |
| Leukocytes[d] (G/L) | 14.7 (6.4) | 14.1 (6.5) | 15.0 (6.8) | NS | 17.5 (8.1) | 13.9 (6.1) | <0.05 | 17.2 (6.2) | 17.8 (9.4) | NS |

Data are presented as mean (SD), number (%) or otherwise stated.
[a]: compares low serum ACE activity with normal serum ACE activity. [b]: compares extremely low serum ACE activity with moderately low serum ACE activity.
[c]: BP was measured in 293 patients, temperature in 294 patients, RR in 229 patients. [d]: Leukocytes in natural log scale for normal distribution in Students'T test.
NS: non significant
BP: blood pressure; RR: respiratory rate; PSI: Pneumonia Severity Index[4]

**Table 2.** Clinical outcome in the subgroups of serum ACE activity

| | | | | | Subdivision of low serum ACE activity group | | |
|---|---|---|---|---|---|---|---|
| | **All patients** n=265 | **Low serum ACE activity < 20 U/L** n=53 | **Normal serum ACE activity >= 20 U/L** n=212 | **p-value[a]** | **Extremely low serum ACE activity < 14 U/L** n=21 | **Moderately low serum ACE activity 14 - 19 U/L** n=32 | **p-value[b]** |
| **Admittance to ICU** | 18 (6.8) | 5 (9.4) | 13 (6.1) | NS | 2 (9.5) | 3 (9.4) | NS |
| **Hospital mortality** | 16 (6.0) | 4 (7.5) | 12 (5.7) | NS | 2 (9.5) | 2 (6.3) | NS |
| **Time to hospital discharge, days** | 10 (6.5) | 11 (8.4) | 10 (6.0) | NS | 10 (9) | 11 (8) | NS |

Data are presented as number (%) or mean (SD).
[a]: compares low serum ACE activity with normal serum ACE activity.
[b]: compares extremely low serum ACE activity with moderately low serum ACE activity.
NS: non significant

**Table 3.** Causative organisms and presence of bacteremia in the subgroups of serum ACE activity

| | | | | | Subdivision of low serum ACE activity group | | |
|---|---|---|---|---|---|---|---|
| | **All patients** n=265 | **Low serum ACE activity < 20 U/L** n=53 | **Normal serum ACE activity >= 20 U/L** n=212 | **p-value[a]** | **Extremely low serum ACE activity < 14 U/L** n=21 | **Moderately low serum ACE activity 14 - 19 U/L** n=32 | **p-value[b]** |
| **Pneumococcal** | 89 (34) | 28 (53) | 61 (29) | <0.01 | 12 (57) | 16 (50) | NS |
| **Atypical** | 49 (19) | 4 (7.5) | 45 (21) | <0.05 | 2 (9.5) | 2 (6.3) | NS |
| **Viral** | 13 (4.9) | 2 (3.8) | 11 (5.2) | NS | 0 (0.0) | 2 (6.3) | NS |
| **Gram negative** | 23 (8.7) | 5 (9.4) | 18 (8.5) | NS | 0 (0.0) | 5 (15.6) | NS |
| **Other** | 10 (3.8) | 2 (3.8) | 8 (3.8) | NS | 0 (0.0) | 2 (6.3) | NS |
| **Unidentified** | 81 (31) | 12 (23) | 69 (33) | NS | 7 (33) | 5 (16) | NS |
| **Bacteremia[c]** | 29 (12) | 13 (27) | 16 (8.5) | <0.01 | 5 (26) | 8 (28) | NS |

Data are presented as number (%).
[a]: compares low serum ACE activity with normal serum ACE activity.
[b]: compares extremely low serum ACE activity with moderately low serum ACE activity.
[c]: blood cultures were taken from 237 patients.
NS: non significant

**Outcome**

None of the clinical outcomes studied showed significant variation between the serum ACE activity strata, as shown in *table 2*. Nevertheless, admittance to ICU and hospital mortality rates were higher and duration of hospital stay was longer in patients with low serum ACE activity. The tendency of worse outcome in the group with low vs. the group with normal serum ACE activities did not continue in the group with extremely low serum ACE activity.

Mortality was 0% for PSI class I and II, 2% for PSI class III, 6 % for PSI class IV and 30% for patients in PSI class V. Systolic blood pressure was not associated with outcome.

**Causative organisms and bacteremia**

In patients with low serum ACE activity, bacteremia was more present (27% versus 9 %, p<0.01) and *S. pneumoniae* was significantly more identified (53% versus 29%, p<0.01) compared with patients with normal serum ACE activity, as shown in *table 3*. In patients with low serum ACE activity, CAP was less frequently caused by atypical organisms (p<0.05). The aetiology of CAP did not differ among the subgroups of extremely and moderately low serum ACE activity.

Because serum ACE activity appeared strongly associated with the presence of bacteremia, an ROC curve was constructed to find the most discriminating value to predict bacteremia. The area under the curve (AUC) was 0.702 (95% CI 0.603 – 0.801, p<0.001). With a cut-off point of <24 U/L, serum ACE activity showed a sensitivity of 69% and specificity of 67% to detect bacteremia. In univariable analysis, serum ACE activity <24 U/L showed an OR of 4.48 (95% CI 1.94 – 10.35) to predict bacteremia. In multivariable analysis, including all other variables associated with bacteremia, this association remained significant. Leukocyte count and CRP level appeared other predictors. The corresponding adjusted OR's were 3.93 (95% CI 1.57 – 9.87) for ACE <24 U/L, 8.10 (95% CI 3.23 – 20.34) for CRP >376 mg/L and 5.44 (95% CI 1.48 – 20.05) for leukocyte count >12.3 G/L (*Table 4*). A sensitivity analysis, in which patients without blood cultures taken were scored as not having bacteremia, confirmed serum ACE activity <24 U/L being indicative for bacteremia (adjusted OR 3.69 (95% CI 1.50 – 9.06)).

**Table 4.** Multivariable regression model of the predictive value of ACE <24 U/L for bacteremia.

| Variable | Effect estimate (β) | Wald statistic | Exp (β) (Odds Ratio) | 95% CI for Exp (β) | p-value |
|---|---|---|---|---|---|
| **ACE <24 U/L** | 1.37 | 8.49 | 3.93 | 1.57-9.87 | <0.01 |
| **CRP >376 mg/L** | 2.09 | 19.84 | 8.10 | 3.23-20.34 | <0.01 |
| **Leukocyte count >12.3 G/L** | 2.70 | 6.50 | 5.44 | 1.48-20.05 | <0.05 |

## Discussion

This study confirmed the strong decrease in serum ACE activity during an episode of pneumonia, but was unable to show a significant prognostic value for ACE activity regarding clinical outcome. Serum ACE activity <24 U/L at time of hospital admission appeared a strong indicator for the presence of bacteremia (adjusted OR 3.93 (95% CI 1.57 – 9.87)). Combined with CRP levels and leukocyte counts, serum ACE activity could become useful to identify bacteremia.

To start, our study provided new insights in the role of ACE during pneumonia. There are different hypotheses that can explain the decrease in serum ACE activity during pneumonia. The first hypothesis is that inflamed (mostly pulmonary) vascular endothelium produces and releases less enzyme. The observation from Al'tshuler *et al*. that serum ACE activity decreased more in patients with polysegmented pneumonia is supportive to such a mechanism.[22] During pneumonia, shedding of ACE from pulmonary vasculature might be impaired, while ACE shedding from other vascular endothelium might be preserved. The present study shows that serum ACE activity decreased more in patients with more systemic inflammation (reflected by high CRP levels, leukocyte counts and bacteremia). In these patients, not only the pulmonary vasculature may be affected, but possibly the entire vascular endothelium. This could explain a stronger decrease in serum ACE activity in septic CAP patients compared to non-septic CAP patients. The ACE secretase that regulates the cleavage of membrane bound ACE into the soluble ACE might play a crucial role in this process. Although the secretase has not yet been identified, it is believed to be a membrane-bound metalloprotease. Metalloproteases are generally elevated in sepsis[23], which at first sight contrasts with the above concept. However, a unifying concept is that after the initial activation of metalloproteases and excessive shedding of membrane-bound ACE, the endothelial pool depletes and serum ACE decreases. Indeed, in pigs made septic by Gram-positive challenge, there was a transient increase in serum ACE activity, followed by a decrease.[24]

A second hypothesis is that during pneumonia and during sepsis in particular, angiotensin II demand increases to preserve homeostasis. The hypotensive state during sepsis can thus, at least in part, be countered by RAAS activation.[25-27] Circulating ACE normally does not affect blood pressure, since only membrane-bound ACE in the (pulmonary) vascular endothelium contributes to angiotensin II production.[28,29] To facilitate a raise in blood pressure during sepsis through increased angiotensin II production, either more renin is released from the kidney, or the amount of membrane bound ACE has to increase. The latter situation might be obtained by reducing ACE secretion from the endothelium.

However, the suggested mechanism does not unambiguously explain why serum ACE activity is decreased in all CAP patients, including patients with normal blood pressure and without signs of sepsis. Possibly this reflects the better ability of these patients to minimize shedding of membrane-bound ACE, thus preserving adequate angiotensin II production and maintaining blood pressure.

A third explanation for the low serum ACE activity during pneumonia could be the presence of a circulating ACE inhibiting compound.[30,31] For example, nitric oxide (NO), NO releasing compounds[32] and reactive oxygen species have been reported to have an inhibitory effect on the activity of ACE[33] and are known to be elevated during sepsis.[34] Conceivably, as *S. pneumoniae* is more identified in patients with low serum ACE activity, the bacterium could produce a toxin, which might function as an exogenous inhibitor of ACE. In order to investigate the possibility of ACE inhibiting substances in the sera with low ACE activity, we conducted an additional dilution curve experiment. Sera from two patients with extremely low serum ACE activity were increasingly diluted (1:1, 1:2, 1:5 and 1:10) with sera from two patients with normal serum ACE activity. Because the obtained dilution curves showed linearity over the entire range (data not shown), the presence of an endogenous or exogenous inhibitor in blood plasma seems unlikely.

A very interesting finding of the present study is the high incidence of bacteremia in patients with low serum ACE activity at time of hospital admission. Several studies have argued for a more aggressive and quick treatment for patients with bacteremia.[35-41] The main advantage of serum ACE activity is that it can be determined quickly at the time of hospital admission and thus allow for more intensive monitoring in contrast to blood cultures that will take longer before they can be interpreted. In our multivariable logistic regression analyses low serum ACE activity (<24 U/L) showed discriminating value for bacteremia above high CRP (>376) and raised leukocyte counts (>12.3). Considering a *pre*-test odds for the presence of bacteremia of 0.14, the likelihood-ratio increased to 7.20 (*post*-test odds: 1.00) when including information about CRP, leukocyte counts and serum ACE activity. In the present study we could not confirm bacteremia as a risk factor for adverse outcome. This null-effect might, however, be due to the thorough microbiological analysis in the present study, as it identified more bacteremia than in other studies.[42]

Besides the hypothesis of the present study that low serum ACE could predict worse outcome in patients with pneumonia, there is also literature suggesting improved outcome for patients on ACE-inhibitor treatment.[43,44] An improved outcome for patients with low ACE activity could have provided interesting leads for study on possible interventions. Because ACE-inhibitor users were

excluded, the association between ACE-inhibitor use and pneumonia outcome has not been addressed in the present study.

There are some limitations to our study. First, we did not measure angiotensin II nor protein levels of ACE to further reveal the pathophysiological processes causing the decrease in serum ACE activity. Furthermore, other biomarkers available such as procalcitonin, pro-adrenomedullin were not measured. Second, the patients of the second cohort were not genotyped for the ACE insertion/deletion polymorphism. In a subgroup analysis, however, the association between serum ACE activity and bacteremia was evenly pronounced when examined in patients of the first cohort with information about genotype available (crude OR = 4.50). More important, it was an *a priori* aim not to include information on ACE genotype in the study because we aimed to explore usefulness of serum ACE activity quantification in a routine care setting. Third, regarding the association between low serum ACE activity and clinical outcome of CAP, we realize that the numbers of worse outcomes in the present study were small and that this could mean that an effect might be missed due to insufficient statistical power. In the present study, the observed 1.3-fold difference in mortality rate between normal and low ACE activity was not statistically significant, but, in our opinion, also not clinically relevant. *Post-hoc*, we calculated that our study had sufficient statistical power to prove a 2.3-fold difference in mortality rate. Finally, one could argue about the serum ACE reference values used. Serum ACE activity is known to differ between populations.[45] In the present study the normal values originated from St. Antonius hospital personnel who volunteered for venapunction. This rules out any geographical or ethnical differences. Also the blood samples of both pneumonia patients and healthy control subjects were analysed in the same laboratory. Furthermore, it is known that ACE levels in the pneumonia patients returned to normal.[6] So, although it is not certain that our cut-off values found also apply to other populations, the abovementioned does imply a large internal validity. The serum activity of our healthy controls is in accordance with other study in white Caucasians.[46]

In conclusion, we were not able to show prognostic value of serum ACE activity in patients with CAP regarding mortality or length of hospital stay. However, we did observe that CAP patients with serum ACE activity <24 U/L at time of hospital admission have an almost fourfold increased risk for the presence of bacteremia compared to patients with normal serum ACE activity. The impact of this finding remains to be elucidated.

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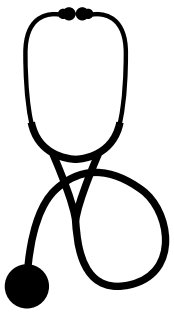

# Chapter 8

# Proteinuria in community-acquired pneumonia as a prognostic marker for outcome

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## Abstract

### Background

Acute kidney injury, classified according to the Risk, Injury, Failure, Loss and End-Stage kidney disease (RIFLE) criteria, is occasionally seen in patients with community-acquired pneumonia. It is known that RIFLE criteria may underestimate the occurrence of acute kidney injury. In this study, we retrospectively investigated the incidence and predictive value of proteinuria as a marker of acute kidney injury in patients with pneumonia and compared the results with the RIFLE criteria.

### Methods

Proteinuria was measured in a urine sample from the day of admission and was defined as total-protein/creatinine ratio >23 mg/mmol. The primary outcome was length of hospital stay. Secondary outcomes were in-hospital mortality and one-year mortality.

### Results

Urine samples were available for 319/496 patients (64%); 198/319 patients (62%) had proteinuria. In univariate analysis, patients with proteinuria had a longer hospital stay (9.0 days [IQR 7.0 – 14.0] versus 7.0 days [IQR 5.0 – 10.0] (Hazard ratio (HR) 1.63, 95% CI 1.28 – 2.06)) and increased in-hospital mortality (OR 5.59, 95% CI 1.27 – 24.63) compared with patients without proteinuria. RIFLE class was a predictor for length of stay (HR 1.55, 95% CI 1.12 – 2.15), in-hospital mortality (OR 3.81, 95% CI 1.40 – 10.35) and one-year mortality (HR 3.06, 95% CI 1.69 – 5.55). In multivariate analysis, only proteinuria was an independent predictor for length of stay.

### Conclusion

The incidence of proteinuria during pneumonia is high and exceeds acute kidney injury classified by the RIFLE criteria. Both proteinuria and RIFLE criteria are associated with adverse outcome in pneumonia. Only proteinuria is an independent predictor for length of hospital stay. Proteinuria can be a marker for outcome and might be used to assess the severity of pneumonia as well.

## Introduction

Acute Kidney Injury (AKI) is a clinical event often seen as a complication in hospitalised patients.[1,2] The occurrence of AKI is classified according to the Risk, Injury, Failure, Loss and End-Stage kidney disease RIFLE criteria, which are based on urine output or a rise in serum creatinine compared to baseline creatinine.[2] The RIFLE criteria are related to adverse clinical outcome in certain contexts such as sepsis.[3] AKI, classified according to the RIFLE criteria, is frequently seen as a complication during community-acquired pneumonia (CAP).[4] The incidence of AKI in CAP ranges in different studies from 18% to 34% and is related to increased mortality.[4,5]

The RIFLE criteria classify patients according to a rise in the serum creatinine level. However, the rise in serum creatinine can be delayed, even after considerable kidney injury.[6] Even small changes in serum creatinine levels predict adverse outcomes.[7] Furthermore, seriously ill patients lose muscle mass, which means that glomerular filtration rate (GFR) has to decline even more before serum creatinine concentration will double.[8] These results suggest that the RIFLE criteria may underestimate the occurrence of AKI. Moreover, a baseline value to reliably assess the change in creatinine is frequently lacking. Taken together, this stresses the need to find other markers for the detection of AKI.[9] Proteinuria is an easily accessible marker of kidney injury.

Proteinuria has been related to both glomerular and tubular dysfunction.[10] Older and recent literature has shown that in patients with chronic kidney disease (CKD) and in the general population, the presence of proteinuria predicts outcome, independent of renal function.[11-15] However, the predictive value of proteinuria during an episode of acute illness has not been reported yet.

In the present study, we investigate the incidence of proteinuria, and the predictive value of proteinuria and the RIFLE criteria on admission in patients admitted with CAP.

## Methods

### Study protocol

This is a retrospective observational analysis of prospectively collected data. We investigated proteinuria and the RIFLE criteria in patients hospitalised with CAP. The RIFLE criteria and proteinuria were related to baseline characteristics and outcome (length of stay (LOS), in-hospital mortality, and one-year mortality).

**Patients**

Data were obtained from patients >18 years of age, who were hospitalised with CAP and who participated in two consecutive clinical trials with similar in- and exclusion criteria.[16,17] Patients were hospitalised during the period from October 2004 to August 2006 or November 2007 to October 2010 in a general 880-bed and a general 550-bed teaching hospital in the Netherlands. In the second cohort, patients were randomised to either dexamethasone or placebo during the first four days of admission.[17] A total of 505 patients were enrolled in these two cohorts. Nine patients on renal replacement therapy were excluded. Selection of the antibiotic treatment was done according to the national guidelines.[18]

**Data collection**

Blood samples were obtained at day 0 (=day of admission) and days 1 to 7 to measure renal function and electrolytes. Urine collection was not part of the study protocols. However, in 238 patients a urine sample, taken on the day of admission, was available. These urine samples were tested for protein, first by standard reagent strip with a threshold of 0.15 mg/L, followed by the laboratorial quantification of total protein. Urine creatinine was measured to correct for concentration. For 81 additional patients, stored frozen (-20˚C) urine samples were available from the day of admission. We measured total protein and creatinine in these samples.

**Proteinuria**

We defined proteinuria as total-protein/creatinine ratio (P/C ratio) of >23 mg/mmol creatinine, following the NFK-KDOQI 2002 guidelines.[19] A P/C ratio in a spot-urine sample is, according to earlier studies, a suitable alternative to the 24-h collection for the quantification of proteinuria.[20]

**RIFLE Criteria**

Patients were classified according to the RIFLE criteria. Baseline serum creatinine values were used if available. For patients in which no known baseline serum creatinine value was available, it was estimated using the 4-variable Modification of Diet in Renal Disease (MDRD) study equation[21], with an assumed eGFR of 75 mL/min/1.73 $m^2$. This approach to estimation has been validated in patients without chronic renal failure.[2] Serum creatinine measured on the day of admission was used to range patients in RIFLE class 'Risk', 'Injury' or 'Failure' if applicable. Patients were classified as class 'Risk' if serum creatinine was 1.5 times baseline creatinine, and class 'Injury' if serum creatinine was twice the baseline value. Class 'Failure' denoted serum creatinine levels that were three-fold higher than the baseline serum creatinine, baseline serum creatinine ≥4

mg/dL (354 µmol/L) with an acute rise >0.5 mg/dL (44 µmol/L). By measuring the urine output over the first several hours, the patient can be reclassified according to the RIFLE criteria. We were not able to reclassify patients as we did not have urine output measures. For this study, the outcome RIFLE categories of loss and end-stage kidney failure were not evaluated. Furthermore, in this study, class 1 refers to class 'Risk', class 2 refers to class 'Injury' and class 3 refers to class 'Failure'.

**Outcomes**

We investigated outcome, with LOS as the primary outcome. The number of days until discharge or death was counted. Secondary outcomes were in-hospital mortality and one-year mortality. One-year mortality was assessed by checking whether the patient visited the hospital after the first year of discharge and if not, the general practitioner of the patient was contacted to ask if the patient was still alive. The date of death was recorded. Four patients did not reach the one-year survival endpoint. Three patients were lost to follow-up.

**Statistical analyses**

All data were analysed with SPSS statistical software for Windows, version 19.0. Categorical data on baseline characteristics were compared using the Chi-square test or Fisher's exact test. Student's t-test was used when data were normally distributed. Non-parametrical data were analysed using the Mann-Whitney U-test. For length of stay, patients who died during their hospital stay were removed from this calculation, because patients who died early would count as having a short length of hospital stay. Cox proportional hazard regression analysis was used for univariate and multivariate analyses of LOS and one-year mortality. For LOS, the event was hospital discharge. Patients who died during hospital stay were censored. Binary logistic regression analysis was used for univariate and multivariate regression analysis of in-hospital mortality. In the multivariate regression analyses, we used the following variables: proteinuria, RIFLE criteria (RIFLE class 0 versus RIFLE classes 1-3), PSI severity (I-III versus IV-V), and dexamethasone. We used Kaplan Meier analysis to visualise the effect of proteinuria and RIFLE criteria on LOS, and of proteinuria and RIFLE classes 1-3 combined on one-year mortality.

We performed a sensitivity analysis in which patients for whom total protein had been quantified using stored frozen urine sample were excluded. The literature shows that urine storage in -20°C can lead to the underestimation of protein quantification.[22]

For all analyses, a p-value of <0.05 was considered statistically significant.

## Results

### Study population

In 319/496 patients (64%) a urine sample was taken on the day of admission. We compared baseline characteristics and outcome in the 319 patients with available urine samples to patients who did not provide a urine sample upon admission. The baseline and outcome table is shown in appendix A. Patients with a urine sample available were slightly older, had more often CKD and had a higher proportion of AKI than those without a urine sample available. Moreover, among patients with a urine sample available, a larger proportion received dexamethasone. One-year mortality was higher in the cohort with a urine sample available compared with the cohort without a urine sample available: 11% versus 6%. This difference was not quite significant (p=0.06). In the 319 patients for whom urine samples were available, 198 (62%) patients had proteinuria (>23 mg/mmol creatinine). The baseline characteristics of subjects with and without proteinuria are shown in *table 1*. All further analyses were performed in the 319 patients for whom urine samples were available.

### Outcome

In this study, median LOS was 8.0 days [IQR 6.0 – 12.0]; 19/319 patients (6%) died during their hospital stay. One-year mortality was 51/312 patients (16%).

### Proteinuria and outcome

The median LOS of patients with proteinuria was 9.0 days [IQR 7.0 – 14.0], as compared to 7.0 days [IQR 5.0 – 10.0] for patients without proteinuria (hazard ratio (HR) 1.63, 95% CI 1.28 – 2.06). Kaplan Meier curve for LOS is shown in *figure 1A*. Proteinuria was associated with increased in-hospital mortality: 17/198 patients (8.6%) with proteinuria died during admission, compared to 2/121 patients (1.7%) without proteinuria (odds ratio (OR) 5.59, 95% CI 1.27 – 24.63). The difference in one-year mortality in patients with proteinuria did not reach statistical difference: 37/193 patients (19%) with proteinuria died, compared to 14/119 patients (12%) without proteinuria (HR 1.74, 95% CI 0.94 – 3.22).

### RIFLE criteria and outcome

Patients in RIFLE classes 1-3 had a median LOS of 9.0 days [IQR 7.0 – 14.0], compared to 8.0 days [IQR 6.0 – 12.0] in patients classified as RIFLE class 0 (HR 1.55, 95% CI 1.12 – 2.15). Kaplan Meier analysis for LOS is shown in *figure 1B*. In-hospital mortality occurred in 7/50 patients (14%) labeled as RIFLE classes 1-3, as compared to 11/268 patients (4.1%) in RIFLE class 0 (OR 3.81, 95% CI 1.40 – 10.35). One-year mortality increased in patients classified as RIFLE classes 1-3 compared to patients labeled as RIFLE class 0: 16/47 patients (34%) in RIFLE

**Table 1.** Baseline characteristics of 319 patients with an available urine sample.

| | Proteinuria n = 198 (62%) | No proteinuria n = 121 (38%) | p-value |
|---|---|---|---|
| **Sex, male** | 113 (57) | 67 (55) | 0.77 |
| **Age, years** | 68.5 [52.5-80.0] | 67.0 [49.0-79.0] | 0.27 |
| **Race**[a] | | | |
| Caucasian | 196 (99) | 119 (98) | 0.62 |
| **Nursing home resident** | 12 ( 6.1) | 4 (3.3) | 0.27 |
| **Comorbidities** | | | |
| CKD | 16 (8.1) | 9 (7.4) | 0.84 |
| Diabetes mellitus | 31 (16) | 20 (17) | 0.84 |
| Liver disease | 0 (0.0) | 2 (1.7) | 0.14 |
| Neoplastic disease | 15 (7.6) | 8 (6.6) | 0.75 |
| Congestive heart failure | 29 (15) | 18 (15) | 0.96 |
| COPD | 26 (13) | 21 (17) | 0.30 |
| **Sepsis (SIRS criteria > 1)** | 171 (90) | 89 (80) | 0.02 |
| **Pneumonia Severity Index class** | | | |
| Class I – III | 96 (49) | 79 (65) | |
| Class IV-V | 102 (52) | 42 (35) | <0.01 |
| **RIFLE class** | | | |
| RIFLE class 0 | 157 (79) | 112 (93) | |
| RIFLE classes 1-3 | 41 (21) | 9 (7.4) | <0.01 |
| **Systemic blood pressure** | | | |
| Systolic (mm Hg) | 132.0 (21.9) | 129.2 (22.7) | 0.28 |
| Diastolic (mm Hg) | 73.2 (11.8) | 74.2 (13.5) | 0.49 |
| **Temperature (°C)** | 38.3 (1.1) | 38.1 (1.1) | 0.03 |
| **Laboratory parameters** | | | |
| C-reactive protein (mg/L) | 264.1 (142.3) | 147.6 (112.4) | <0.01 |
| White blood count (x109/L) | 15.3 (6.8) | 13.8 (6.5) | 0.06 |
| Creatinine (µmol/L) | 91.0 [72.0-126.5] | 83.0 [69.0-108.5] | <0.01 |
| Blood urea nitrogen (mg/dL) | 23.5 [14.8-35.3] | 17.6 [11.5-24.9] | <0.01 |
| **Current medication** | | | |
| NSAID | 20 (10) | 16 (13) | 0.38 |
| ACE inhibitor or ARB | 36 (18) | 31 (121) | 0.11 |
| **Dexamethasone**[b] | 60 (30) | 56 (46) | <0.01 |

Data are presented as mean, median [IQR] or number (%). Abbreviations: ACE: angiotensin-converting enzyme, ARB: angiotensin receptor blocker, CKD: chronic kidney disease, COPD: chronic obstructive pulmonary disease.
[a]: Race was self-reported. [b]: As part of a clinical trial.

classes 1-3 died compared to 34/264 (13%) of the patients in RIFLE class 0 (HR 3.06, 95% CI 1.69 – 5.55).

To account for a possible impact of selection bias on our results, we analysed the predictive value of the RIFLE criteria for the patient cohort that was not included in the study as a result of unavailability of a urine sample. In this cohort, the RIFLE criteria did predict the LOS, adjusted and unadjusted. Neither

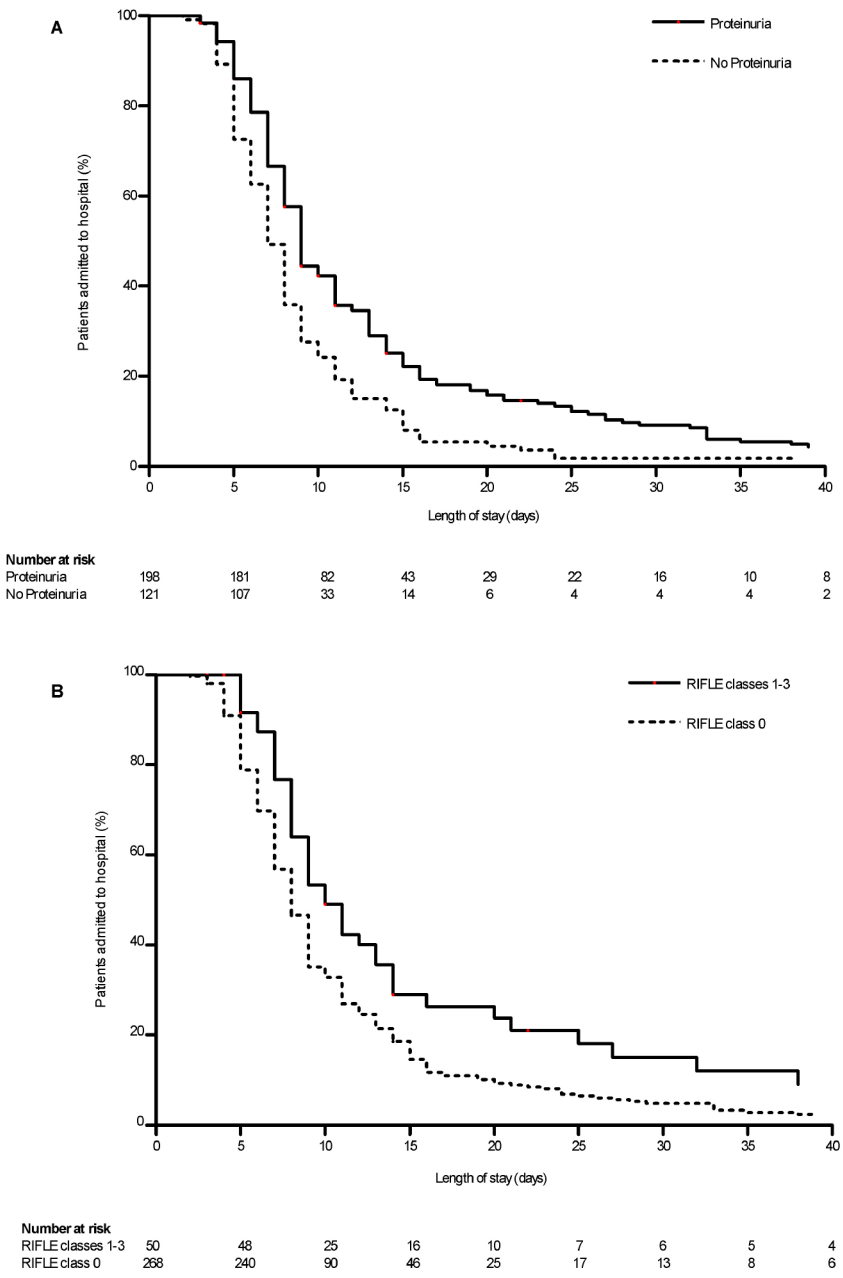



**Figure 1.** Kaplan Meier analysis of length of stay in patients with community-acquired pneumonia
A: The effect of proteinuria on length of stay (log-rank p<0.001).
B: The effect of RIFLE classification on length of stay (log-rank p=0.005).

in-hospital mortality nor one-year mortality was predicted by the RIFLE criteria. Odds and hazard ratios are shown in appendix B.

**Proteinuria versus RIFLE criteria**

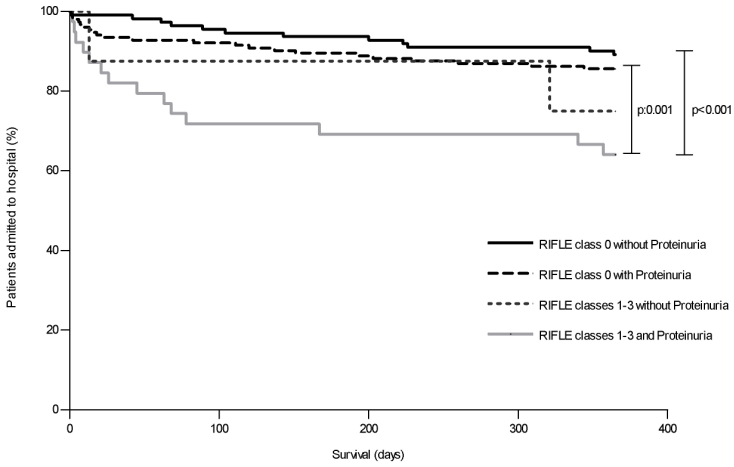
Proteinuria was an independent predictor for LOS: HR 1.38, 95% CI 1.08 – 1.75. RIFLE criteria appeared not to be an independent predictor (HR 1.21, 95% CI 0.86 – 1.71). The administration of dexamethasone and PSI score were also independent predictors of LOS in this model. For in-hospital mortality, proteinuria had an OR 3.27, 95% CI 0.69 – 15.45 and RIFLE criteria had OR 1.81, 95% CI 0.62 – 5.27. We compared RIFLE criteria with proteinuria as predictor for one-year mortality. Neither proteinuria nor RIFLE criteria were independent predictors when PSI severity (I-III versus IV-V) and dexamethasone were in the model.

We performed the same analyses for the combined variable proteinuria and RIFLE classes 1-3. *Figure 2* shows Kaplan Meier analysis of one-year survival of the cohort, stratified in four subgroups (neither RIFLE classes 1-3 nor proteinuria, either RIFLE classes 1-3 or proteinuria, or both RIFLE classes 1-3 and proteinuria). All univariate analyses were significant in contrast to all multivariate results of this combined variable. All odds and hazard ratios can be found in *table 2*.

**Table 2.** Odds ratios and hazard ratios for all outcomes, classified according to proteinuria, RIFLE classes 1-3 or proteinuria and RIFLE classes 1-3 combined

| | Proteinuria | | RIFLE classes 1-3 | | Proteinuria + RIFLE classes 1-3 | |
|---|---|---|---|---|---|---|
| | OR or HR (95% CI) | p-value | OR or HR (95% CI) | p-value | OR or HR (95% CI) | p-value |
| **Length of stay** | | | | | | |
| Unadjusted[2] | 1.63 (1.28-2.06) | <0.01 | 1.55 (1.12-2.15) | <0.01 | 1.69 (0.41-0.85) | <0.01 |
| Adjusted[2] | 1.38 (1.08-1.75) | 0.01 | 1.21 (0.86-1.71) | 0.27 | 1.37 (0.51-1.06) | 0.09 |
| **In-hospital mortality** | | | | | | |
| Unadjusted[1] | 5.59 (1.27-24.63) | 0.02 | 3.81 (1.40-10.35) | <0.01 | 3.79 (1.34-10.72) | 0.01 |
| Adjusted[1] | 3.27 (0.69-15.45) | 0.14 | 1.81 (0.62-5.27) | 0.28 | 1.90 (0.63-5.73) | 0.25 |
| **One-year mortality** | | | | | | |
| Unadjusted[2] | 1.74 (0.94-3.22) | 0.08 | 3.06 (1.69-5.55) | <0.01 | 3.21 (1.73-5.95) | <0.01 |
| Adjusted[2] | 1.09 (0.56-2.10) | 0.80 | 1.81 (0.97-3.37) | 0.06 | 1.80 (0.94-3.42) | 0.08 |

Unadjusted and adjusted odds ratios[1] and hazard ratios[2]. Adjusted ratios are calculated for proteinuria and RIFLE classes 1-3 together with PSI severity (I-III versus IV-V) and dexamethasone in one model, or proteinuria + RIFLE classes 1-3 together with PSI severity and dexamethasone in one model. Abbreviations: CI = confidence interval, HR = hazard ratio, OR = odds ratio.




**Number at risk**

| | | | | |
|---|---|---|---|---|
| RIFLE class 0 without Proteinuria | 111 | 107 | 105 | 102 |
| RIFLE class 0 with Proteinuria | 153 | 142 | 137 | 134 |
| RIFLE classes 1-3 without Proteinuria | 8 | 8 | 8 | 8 |
| RIFLE classes 1-3 and Proteinuria | 39 | 29 | 28 | 28 |

**Figure 2.** Kaplan Meier analysis of the effect of proteinuria and RIFLE combined on one-year mortality (log-rank p<0.001).

### Sensitivity analysis

In a sensitivity analysis, patients in which total protein was quantified using a stored frozen urine sample (n=81) were excluded. Analyses of the other 238 patients did not change the results of the analyses in the full dataset. Outcomes are shown in appendix C.

## Discussion

In this study, we observed that proteinuria is frequently present in patients with CAP. Its occurrence is substantially higher than the occurrence of AKI according to the RIFLE criteria. Proteinuria was independently associated with a longer length of hospital stay; the RIFLE criteria were not. For in-hospital mortality, proteinuria had a higher adjusted OR compared to the RIFLE criteria (3.27 versus 1.81), however this was not statistically significant. For proteinuria and RIFLE classes 1-3 combined, the univariate analyses were significant for all outcomes. Though, in multivariate analyses, this effect was lost.

In this study, 16% of the patients were staged as RIFLE classes 1-3. This percentage is consistent with the literature on CAP.[5] The RIFLE criteria have been shown to be related with an increased mortality in several groups of patients, e.g. in patients with severe and non-severe CAP.[4] In agreement with these studies, we also observed increased mortality for patients staged in RIFLE classes 1-3

in univariate analysis. Contrary to previous studies, in our study, the RIFLE criteria were not an independent predictor for in-hospital mortality or one-year mortality when corrected for PSI score.[4] Former studies have demonstrated that proteinuria, when present in healthy individuals, can be a predictor for the development of AKI and increased mortality.[11] Albuminuria was proposed as a useful biomarker of AKI in two studies, based mainly on investigations in mice and rats.[23,24] We believe this is the first study that investigates the predictive value of proteinuria during acute infection in a clinical setting.

Data on proteinuria during sepsis are sparse, however, micro-albuminuria is frequently seen in septic patients.[25-27] In the current study, proteinuria was present in 62% of the patients in whom a urine sample was available, as opposed to AKI classified according to the RIFLE criteria in 16% of the patients. Among the 50 patients with RIFLE classes 1-3, 41 had proteinuria, therefore AKI is often associated with proteinuria. On the other hand, proteinuria without AKI occurred in 156 patients. Pathophysiologic changes occurring during inflammatory disorders can affect glomerular permeability, due to the presence of pro-inflammatory cytokines.[10] Proteinuria may also result from a defect in proximal tubular reabsorption.[10]

This study has several strengths. First, a urine quantification measurement is easy to obtain in every patient. This is in contrast with the RIFLE class, as baseline values are often not available. Second, urine P/C ratio quantification is an inexpensive test, in contrast with other recently introduced urinary markers for AKI, such as kidney injury molecule-1, cystatin C and neutrophil-gelatinase-associated lipocalin.[9] Third, the Acute Kidney Injury Network (AKIN) proposed a refinement of the RIFLE criteria, through use of dynamic changes in serum creatinine during the first 48 hours of admission to classify patients with AKI.[28] Use of this method reduced the need for a baseline creatinine value. This classification method did not seem to improve sensitivity or predictive value compared to the RIFLE criteria[29]; therefore we limited our analysis to comparison with the classical RIFLE criteria. Fourth, only three patients (0.9%) were lost to follow-up and could not be included to calculate one-year mortality.

Our study has limitations. Only 319/496 (64%) of the patient population who had a useful urine sample available, could be included. Patients with a urine sample were more often staged in RIFLE classes 1-3, were slightly older, were more often known with CKD, and less often with COPD. Thus, availability of a urine sample was not random, and selection may have occurred. This could have had an effect on the high prevalence of proteinuria found in our study. Because the predictive value of presence of proteinuria was investigated, this probable confounder does not challenge the conclusion that proteinuria predicts a longer length of stay. Also, there was no significant difference in

outcomes between these cohorts. Furthermore, we analysed the predictive value of the RIFLE criteria in the cohort of patients without a urine sample. The RIFLE criteria predicted independently LOS in those patients, but did also not predict in-hospital mortality nor one-year mortality.

A second limitation was that a baseline creatinine value was not available in all patients. In patients in which a baseline creatinine value was not available, an estimated baseline creatinine was back-calculated by using an eGFR of 75 mL/min/1.73 $m^2$, as recommended by the ADQI group.[2] This could have resulted in underperformance of the RIFLE criteria for predicting outcome. Third, in this study, we used the total-protein/creatinine, with a threshold of 23 mg/mmol creatinine. This was recommended by the NFK-KDOQI guidelines from 2002.[19] The NFK-KDOQI guidelines from 2007 do not indicate a particular P/C ratio threshold value. We decided to use the threshold of 2002, as it is the lowest value specified in guidelines. Micro-albuminuria and albumin/creatinine ratio (A/C ratio) might be more sensitive compared to P/C ratio.[30] The SIGN CKD guidelines from 2008 state that both P/C ratio and A/C ratio are accurate methods with which to rule out proteinuria, when the probability of proteinuria is high.[31] Furthermore, baseline values of urinary protein excretion were not available. Therefore it was not possible to distinguish between acute and chronic proteinuria. The presence of proteinuria in 62% of patients (a percentage that is much higher than the presence of proteinuria in the general population[32]) suggests that the observed proteinuria results from CAP in most patients. We were not able to obtain any urinary protein measurements obtained after the CAP episode either. It would be interesting to see whether the proteinuria was transient or persistent. The course of kidney injury during CAP requires further investigation.

Finally, the data were used to investigate the influence of dexamethasone on LOS in patients hospitalised with CAP.[17] Patients who were included in our analysis received dexamethasone more frequently. This intervention did not bias the baseline data, as the conclusion was based on urine samples that were obtained before the administration of dexamethasone or placebo. Patients without proteinuria received dexamethasone more often compared to patients with proteinuria. This could have influenced the longer LOS found for patients with proteinuria. We corrected for the use of dexamethasone in multivariate analyses on outcome.[17]

In conclusion, proteinuria is common in patients with CAP. In contrast to the RIFLE criteria, proteinuria is an independent predictor for length of hospital stay. Proteinuria can be a marker for outcome, with the advantage of circumventing the need for a baseline sample, and may be used to assess the severity of CAP as well.

# Appendix A

Baseline characteristics and outcome of 496 patients with community-acquired pneumonia

| | Urine sample n = 319 (64) | No urine sample n = 177 (36) | p-value |
|---|---|---|---|
| **Sex, male** | 180 (56) | 111 (63) | 0.17 |
| **Age, years** | 68.0 [51.0-80.0] | 65.0 [51.0-74.0] | 0.05 |
| **Race**[a] | | | |
| Caucasian | 315 (99) | 176 (99) | 0.66 |
| **Nursing home resident** | 16 (5.0) | 3 (1.7) | 0.07 |
| **Comorbidities** | | | |
| CKD | 25 (7.8) | 6 (3.4) | 0.05 |
| Diabetes mellitus | 51 (16) | 22 (12) | 0.28 |
| Liver disease | 2 (0.6) | 0 (0.0) | 0.54 |
| Neoplastic disease | 23 (7.2) | 22 (12) | 0.05 |
| Congestive heart failure | 47 (15) | 19 (11) | 0.21 |
| COPD | 47 (15) | 49 (28) | <0.01 |
| **Sepsis (SIRS criteria > 1)** | 260 (86) | 142 (85) | 0.75 |
| **Pneumonia severity index class** | | | |
| Class I – III | 175 (55) | 101 (57) | |
| Class IV-V | 144 (45) | 76 (43) | 0.64 |
| **RIFLE class** | | | |
| RIFLE class 0 | 268 (84) | 163 (92) | |
| RIFLE classes 1-3 | 50 (16) | 14 (7.9) | 0.01 |
| **Systemic blood pressure** | | | |
| Systolic (mm Hg) | 131.0 (22.2) | 135.2 (23.2) | 0.05 |
| Diastolic (mm Hg) | 73.5 (12.5) | 75.1 (14.0) | 0.20 |
| **Temperature (°C)** | 38.2 (1.1) | 38.1 (1.1) | 0.27 |
| **Laboratory parameters** | | | |
| C-reactive protein (mg/L) | 219.9 (143.2) | 209.7 (136.0) | 0.44 |
| White blood count (x $10^9$/L) | 14.7 (6.7) | 14.3 (6.4) | 0.50 |
| Creatinine (μmol/L) | 89.0 [71.0-117.3] | 90.0 [72.0-107.0] | 0.74 |
| Blood urea nitrogen (mg/dL) | 20.4 [13.2-30.8] | 17.6 [13.7-26.6] | 0.07 |
| **Current medication** | | | |
| NSAID | 36 (11) | 16 (9.1) | 0.43 |
| ACE inhibitor and/or ARB | 67 (21) | 38 (22) | 0.90 |
| **Dexamethasone**[b] | 116 (36) | 31 (18) | <0.01 |
| **Outcome** | | | |
| Length of stay | 8.0 [6.0-13.0] | 9.0 [6.0-14.0] | 0.31 |
| In-hospital mortality | 19 (6.0) | 5 (2.8) | 0.13 |
| One-year mortality | 51 (16) | 19 (11) | 0.06 |

Data are presented as mean (SD), median [IQR] or number (%). Abbreviations: ACE: angiotensin-converting enzyme inhibitor, ARB: angiotensin receptor blocker, CKD: chronic kidney disease, COPD: chronic obstructive pulmonary disease.
[a]: Race was self-reported. [b]: As part of a clinical trial.

## Appendix B

Odds ratios and hazard ratios for all outcomes, classified according to RIFLE classes 1-3, for patients without a urine sample available (n=177)

| | RIFLE classes 1-3 | | |
|---|---|---|---|
| | **OR or HR** | **95% CI** | **p-value** |
| **Length of stay** | | | |
| Unadjusted[2] | 3.62 | 1.88-6.97 | <0.01 |
| Adjusted[2] | 4.15 | 2.15-8.02 | <0.01 |
| **In-hospital mortality** | | | |
| Unadjusted[1] | 2.84 | 0.30-27.17 | 0.37 |
| Adjusted[1] | 3.65 | 0.33-40.90 | 0.30 |
| **One-year mortality** | | | |
| Unadjusted[2] | 2.32 | 0.67-8.02 | 0.18 |
| Adjusted[2] | 2.66 | 0.77-9.22 | 0.12 |

Unadjusted and adjusted odds ratios (OR=[1]) and hazard ratios (HR=[2]) for RIFLE classes 1-3. Adjusted ratios are calculated for RIFLE classes 1-3 together with proteinuria, PSI severity (I-III versus IV-V) and dexamethasone in one model.

## Appendix C

Odds ratios and hazard ratios for all outcomes of the sensitivity analysis in 238 patients

| | Proteinuria | | | RIFLE classes 1-3 | | |
|---|---|---|---|---|---|---|
| | **OR or HR** | **95% CI** | **p-value** | **OR or HR** | **95% CI** | **p-value** |
| **Length of stay** | | | | | | |
| Unadjusted[2] | 1.72 | 1.30-2.26 | <0.01 | 1.46 | 1.02-2.09 | 0.04 |
| Adjusted[2] | 1.49 | 1.12-1.98 | <0.01 | 1.19 | 0.81-1.73 | 0.38 |
| **In-hospital mortality** | | | | | | |
| Unadjusted[1] | 9.67 | 1.25-74.50 | 0.03 | 3.89 | 1.36-11.10 | 0.01 |
| Adjusted[1] | 6.39 | 0.79-51.59 | 0.08 | 1.80 | 0.58-5.53 | 0.31 |
| **One-year mortality** | | | | | | |
| Unadjusted[2] | 1.82 | 0.92-3.62 | 0.09 | 3.89 | 1.39-5.01 | <0.01 |
| Adjusted[2] | 1.30 | 0.63-2.68 | 0.48 | 1.52 | 0.77-2.98 | 0.23 |

Unadjusted and adjusted odds ratios (OR=[1]) and hazard ratios (HR=[2]) for the presence of proteinuria and RIFLE classes 1-3. Adjusted ratios are calculated for proteinuria and RIFLE classes 1-3 together with PSI severity (I-III versus IV-V) and dexamethasone in one model.

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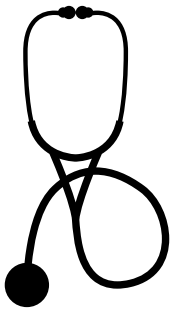
# Part 4

## Adjunctive therapy



# Chapter 9

# Therapy in pneumonia: What is beyond antibiotics?

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## Abstract

Community-acquired pneumonia (CAP) is a common and serious disease with significant mortality, morbidity and associated healthcare costs. Severity of pneumonia is related to the extend of the inflammatory response. Primary goal in the treatment of pneumonia is starting adequate antibiotic therapy as soon as possible. However, antimicrobial resistance among the most common bacteria causing pneumonia is increasing. For those two reasons, extended inflammatory response and increasing antibiotic resistance, it is interesting to look at adjunctive non-antibiotic therapeutic strategies aimed at modulation of the inflammatory response or at the microorganism itself. In this review, we discuss the current knowledge regarding these therapies and their possible role in the future.

## Introduction

Community-acquired pneumonia (CAP) is the most common infectious disease that necessitates hospitalisation.[1] Hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) are serious complications of hospital stay and mechanical ventilation, respectively.[2,3] Despite advances in prevention by vaccination, microbiological diagnostics and antibiotic therapy, pneumonia is still characterised by a high mortality and morbidity and is associated with significant healthcare costs.[4,5] The mainstay of CAP therapy is early diagnosis and initiation of appropriate antibiotic therapy within four hours to minimize the door-to-needle time.[6] Antibiotic therapy for HAP and VAP is even more challenging due to the increase in antibiotic resistance of Gram-negative bacteria.[7] Unfortunately, there is also a trend of increasing antibiotic resistance in the most common bacteria that cause CAP, *Streptococcus pneumoniae* and *Haemophilus influenzae*.[8,9] Despite adequate antibiotic therapy, a substantial number of patients at high risk of deterioration require additional therapies for pneumonia. These therapies are aimed either at the microorganism or at the host. Therapeutic targets are improvement of recognition of microbial antigens (with complement or Toll-like receptors), improvement of effector mechanisms of the immune response (with immunoglobulins) and limiting immunopathology caused by the cytokine storm (with corticosteroids, statins or activated protein C (APC)). Certain antibiotics, such as macrolides, also can limit the damage caused by an overactive immune system. We will limit this review to treatment options for an immunocompetent hospitalised patient receiving appropriate antibiotics.

## Therapy targeted at improvement of bacterial opsonisation

### Complement cascade

The complement system can be activated in three ways: classical pathway activation after antibodies have been bound to the microorganisms, alternative pathway activation, and activation by mannose residues (mannose-binding lectin (MBL) route). Complement activation via either route ultimately results via a cascade of steps in the formation of a membrane-attack complex, which results in lysis of the pathogen. Other complement split products are deposited on the surface of the microorganism which promote its phagocytosis. MBL binds to several respiratory pathogens including *Haemophilus influenzae*[10,11], *Mycoplasma pneumoniae*[12] and *Legionella pneumophila*[13], and to a lesser degree *Streptococcus pneumoniae*.[14]

Polymorphisms in the structural and promoter sequences of the MBL2 gene lead to a deficiency in MBL production, with a frequency of approximately 10-15% in the normal healthy population.[15] Susceptibility to lower respiratory tract infections seems not to be affected by MBL deficiency.[16-18] However, MBL deficiency is associated with a more severe clinical course of pneumonia and the development of more severe forms of sepsis, ICU admission and fatal outcome in lower respiratory tract infections.[19,20]. Also in invasive pneumococcal disease, several studies found an increased frequency of MBL deficiency.[21-23].

Thus far, MBL substitution therapy has only been tested in phase I and II trial, and to date no negative clinical effects are reported.[24,25] However, over substitution should be avoided because high MBL genotypes are associated with nephropathy in patients with diabetes mellitus type 1 and vascular tissue damage in myocardial ischemia-reperfusion injury.[26,27] To date, MBL replacement has not been used in pneumonia patients. MBL substitution might be of value as adjunctive therapy for MBL deficient patients.

**Toll-like receptors**

Toll-like receptors (TLRs) are a family of receptors that activate the inflammatory response after recognition of molecular patterns that are present on different pneumonia-associated microorganisms.[28,29] The role of TLRs in sepsis has been recently reviewed.[30,31] Polymorphisms in the genes coding for TLRs are associated with increased susceptibility to (severe) sepsis, including pneumonia or sepsis caused by *S. pneumoniae*.[32,33] Because they are a major trigger for the inflammatory response, TLRs are regarded as a promising target for anti-inflammatory therapy.

In an animal model for pneumococcal pneumonia, triggering of TLR5 with its ligand, flagellin, leads to a substantial better survival.[34] This shows the importance of the immediate activation of the innate response in clearance of a pulmonary infection.

As indicated above, over activation of the inflammatory response can cause substantial damage and should therefore be avoided. TAK-242 is an agonist of another TLR, TLR4, and inhibits intracellular signalling and thereby preventing up-regulation of the inflammatory response. TLR4 is a lipopolysaccharide (LPS) binding receptor, and Gram-negative LPS containing bacteria are a major cause of severe sepsis in critically ill patients. TLR4 is the only receptor in which blocking seems an interesting additive therapy.[35,36] The first recently published double-blind randomised trial comparing TAK-242 to placebo in patients with severe sepsis and septic shock did not shown a difference in mortality. Furthermore, treatment with TAK-242 did not succeed in decreasing cytokine

levels, which suggests that other inflammatory pathways are involved.[37] These studies have been performed in critically ill patients, and subgroup analyses of patients with pneumonia are lacking. Furthermore, TLR4 is mainly involved in the inflammatory response to LPS-containing bacteria, and these bacteria are uncommon in community-acquired pneumonia.
Currently, there is no role for TLR antagonists in the treatment of pneumonia, or severe sepsis or septic shock.

## Therapy targeted at improvement of effector mechanisms of the immune response

### Immunoglobulins

In the period before antibiotics were available (up to 1940) treatment of pneumococcal pneumonia consisted of the passive administration of serotype specific antibodies.[38] Nowadays, substitution therapy with immunoglobulins is used for long-term treatment of patients with humoral immunodeficiency diseases.[39,40] By replacing or increasing, the levels of immunoglobulins, especially Immunoglobulin G (IgG), the inflammatory response to the bacteria could be improved by trapping endotoxins and facilitating phagocytosis. Clinical studies on the use of intravenous immunoglobulins IgG (IVIG) in infectious disease are limited and mainly focused on patients with streptococcal toxic shock syndrome and severe sepsis and septic shock.[41] Although consecutive reviews showed improved outcome of patients treated with IVIG, the use of immunoglobulins in critically ill patients is still controversial. [42-45] It is unclear whether the benefit of IVIG therapy was due to the antibody concentration or to volume resuscitation with proteins, or to an anti-inflammatory effect.[46] All studies contained numerous patients with severe sepsis or septic shock due to pneumonia, but there were no subgroup analyses investigating the effect of IVIG in patients with pneumonia. Therefore, immunoglobulins for pneumonia in general remains unclear and their use remains restricted to patients with severe sepsis or septic shock.

## Therapy targeted at limiting immunopathology

### Corticosteroids

The inflammatory cytokine response in the lung is characterised by a short intense elevation in TNF-α followed by increases in IL-1β and IL-6. A subsequent increase in IL-10, which is an anti-inflammatory cytokine that inhibits macrophage and neutrophil production, is the beginning of the anti-inflammatory response that prevents an uncontrolled inflammatory response.[47-49] In pneumonia patients, this hierarchy of cytokine response is not observed, because the

inflammatory response is already ongoing upon admission to the hospital.[50] In most patients these cytokines control and eliminate the primary infection; however, in some patients, the cytokine activation becomes widespread. This indicates the need for a delicate balance between a sufficient and excessive cytokine response. The extended systemic inflammatory response is presumed to play a role in the organ dysfunction that is characteristic of severe sepsis and septic shock.[51] Among patients with pneumonia, non-survivors of CAP exhibit persistent elevation in inflammatory cytokine levels over time, compared to survivors.[50,52] Modulation of this inflammatory response during infection is an attractive concept.

Corticosteroids are the most important physiological inhibitors of inflammation. They can switch off genes that encode pro-inflammatory cytokines and switch on those that encode anti-inflammatory cytokines.[53] Prolonged (>5 days) treatment with low-dose corticosteroids can down-regulate inflammatory cytokine transcription and accelerate the resolution of critical illness.[54] In addition to this direct effect on gene transcription, recent observations have shown that corticosteroids might be effective in patients with severe sepsis due to relative adrenal insufficiency associated with critical-illness and systemic inflammation induced glucocorticoid receptor resistance  Not only in severe sepsis and septic shock, but also in pneumonia there are different reasons in support of a beneficial effect of corticosteroids. [55,56] Corticosteroids might be effective in reducing excessive pulmonary inflammation and thereby reducing lung injury.[57] Furthermore, in some cases of pneumonia, bronchospasm can play a significant role (e.g., in patients with COPD/asthma or viral-induced pneumonia), which can be counteracted by corticosteroids.[58,59]

Over the last several decades, corticosteroids have been used as adjunctive therapy in patients with sepsis and septic shock. Initial trials investigating short courses of high doses found no beneficial effect on mortality, whereas more recent trials showed that a low dose (< 300 mg/d) of hydrocortisone for a longer duration (>5 days) may improve survival.[60-63]
In contrast to this large number of studies on sepsis and septic shock, randomised controlled trials (RCT) using corticosteroids as an adjunctive treatment to antibiotics in pneumonia are limited and have variable results. The use of corticosteroid treatment in CAP dates back to 1956, when favourable effects of hydrocortisone in patients with pneumococcal pneumonia were reported.[64] Two more recent studies found a significant reduction in hospital mortality and length of hospital stay in patients with severe CAP who were treated with adjunctive corticosteroids. Confalonieri *et al*. found a marked

improvement in the ratio of the partial pressure of oxygen in arterial blood ($PaO_2$) to the fraction of inspired oxygen ($FiO_2$) as well as a survival advantage in patients with severe CAP treated with hydrocortisone for 7 days. A retrospective study showed that patients with severe CAP who were treated with systemic corticosteroids had a reduced risk of mortality compared to patients without adjunctive corticosteroids.[65] A smaller randomised controlled trial compared prednisolone for 3 days with a placebo in patients with CAP of any severity and found no effect on hospital stay; however, in patients with moderate or severe CAP, corticosteroids promoted resolution of clinical symptoms and reduced the duration of intravenous antibiotic treatment.[66] To date, a recent study by Snijders *et al*. is the largest to evaluate the role of prednisolone in patients with CAP of any severity.[67] In that RCT no beneficial effects of adjunctive corticosteroids on CAP were found.

There may be some potential adverse effects of the use of corticosteroids for CAP. From a theoretical point of view, the risk of gastrointestinal bleeding, muscle weakness and metabolic disorders is increased. In addition, down-regulation of the host response to infection might increase the risk of nosocomial infections and reactivation of viral infections. In a systematic review of twenty RCTs that involved adjunctive corticosteroid therapy in sepsis, these serious adverse events did not occur more often than in placebo-treated patients. However, hyperglycaemia and hypernatraemia were observed more frequently in the corticosteroid-treated patients.[68]

**Statins**

In addition to modulation of the inflammatory response by corticosteroids, in experimental studies statins have shown to have significant anti-inflammatory properties.[69] These benefits are not ascribed to their cholesterol-lowering activity but rather to a pleiotropic effect on isoprenoid synthesis that results in the down-regulation of intracellular inflammatory signalling; this leads to modulation of the immune response, which results in a reduction in cytokine levels.[70] Moreover, statins improve endothelial function and may modify the balance of coagulation towards a less prothrombotic state, as seen in sepsis. Large retrospective observational studies have shown a potential positive effect on mortality in patients with severe infections or sepsis.[71,72] However, in a prospective cohort study, statins were not associated with reduced mortality or less ICU admissions.[73] Large RCTs are needed to evaluate the effect of an intervention with statin therapy during CAP.

**Activated protein C**

An exaggerated inflammatory response can result in a decline in Protein C, which is a soluble anticoagulant and prefibrinolytic enzyme. Reduced levels of activated protein C (APC), which leads to a procoagulant state, are associated with an increased risk of death in septic patients.[74] In patients with severe sepsis, APC has been shown to reduce mortality (PROWESS trial).[75] This may be due to its anticoagulant activity, but there is also evidence that APC is an inhibitor of the systemic inflammatory response.[76] In a subanalysis of the PROWESS trial, a survival benefit was seen in patients with CAP-associated sepsis and a high mortality risk (APACHE >25) who were treated with APC compared to placebo.[77] However, administration of APC increases the risk of serious bleeding, with reported rates of up to 10%.[78] Therefore, recent guidelines recommend that APC should only be considered in patients with severe sepsis and a high risk of death but not in the overall CAP population.[79]

**Macrolide antibiotics**

Several antibiotics that are used in the therapy of CAP appear to have actions beyond direct antibacterial activities. Macrolides are known to also posses immunomodulatory effects.[80] Macrolides accumulate in inflammatory cells and modulate their actions, which results in modification of leukocyte function, cytokine expression and mucus production. Macrolides infer a biphasic effect on the host. First, it has direct antimicrobial activity by stimulating the host defence against bacteria via stimulation of leukocyte degranulation, phagocytosis and oxidative burst. Second, after the acute infection, neutrophils that are primed by cytokines or LPS are inhibited by macrolides, which leads to resolution of the inflammatory response. Moreover, macrolides may also improve macrophage function, which results in the increased removal of apoptotic debris.[81] Another potential explanation for the beneficial effects of macrolides is reduction in bacterial load with less cell wall lysis than beta-lactam antibiotics; this results in a more gradual reduction in bacterial load and, therefore, a more gradual release of immunologically reactive components, which may prevent an extended systemic inflammatory response.

The beneficial effect of macrolides has been recognised in chronic pulmonary diseases, probably through inhibition of quorum-sensing in biofilms, but some studies found improved outcomes in CAP patients who were treated with macrolide-containing antibiotic regimes.[82,83] The outcome in pneumococcal pneumonia was improved when a macrolide was added to standard treatment, even when the bacteria was sensitive to standard treatment.[84,85] This effect appears to be most prominent in severe bacteraemic pneumococcal

pneumonia.[86] However, other studies were unable to show a beneficial effect of macrolides in CAP.[87,88]

## Conclusion

At this moment, timely administration of appropriate antibiotics is still the most important therapy in pneumonia.[89] Beyond antibiotics, there are other targets for adjunctive therapy. For immunoglobulins, APC and TLR4-antagonists, the majority of evidence is extrapolated from studies on severe sepsis and septic shock. Many patients in these studies suffered from pneumonia, but only in a part of these studies reliable subgroup analysis was performed. Furthermore, results from these studies are conflicting and most meta-analyses do not provide firm conclusions. The only conclusion that can be drawn is that immunoglobulins are a promising therapy in patients with pneumonia and severe sepsis or septic shock. APC might be used in patients with pneumonia and severe sepsis or septic shock with a APACHE-score (>25). To date, for the patient with CAP there is no role for therapy with TLR4-antagonists or MBL. Adding macrolides to the antibiotic regimen is an interesting and promising strategy, but prospective RCTs are necessary. Currently, there is consensus on the use of corticosteroids in septic shock. Nevertheless, the use of corticosteroids in patients with pneumonia without severe sepsis or septic shock is still unclear, but the results of new studies will be reported in the near future.

In conclusion, in this review we have discussed the various options for supportive therapy of patients who are treated with otherwise effective antibiotics. In view of increasing resistance, these supportive therapies might become the only option left. However, neither corticosteroids, nor APC, immunoglobulins or any of the others could probably be used as monotherapy. As adjunctive therapy so far, corticosteroids, APC, and immunoglobulins are available and can be used in patients with CAP complicated by severe sepsis or septic shock. Complement, including MBL and TLR agonists and antagonists are attractive options but warrant additional studies because insufficient evidence is available to date.

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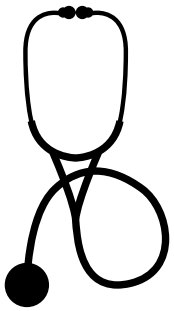

# Chapter 10

# Dexamethasone and length of hospital stay in patients with community-acquired pneumonia: a randomised, double-blind, placebo-controlled trial

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## Abstract

### Background

Whether addition of corticosteroids to antibiotic treatment benefits patients with community-acquired pneumonia who are not in intensive care units is unclear. We aimed to assess effect of addition of dexamethasone on length of stay in this group, which might result in earlier resolution of pneumonia through dampening of systemic inflammation.

### Methods

In our double-blind, placebo-controlled trial, we randomly assigned adults aged 18 years or older with confirmed community-acquired pneumonia who presented to emergency departments of two teaching hospitals in the Netherlands to receive intravenous dexamethasone (5 mg once a day) or placebo for 4 days from admission. Patients were ineligible if they were immunocompromised, needed immediate transfer to an intensive-care unit, or already receiving corticosteroids or immunosuppressive drugs. We randomly allocated patients on a one-to-one basis to treatment groups with a computerised randomisation allocation sequence in blocks of 20. The primary outcome was length of hospital stay in all enrolled patients.

### Results

Between November, 2007, and September, 2010, we enrolled 304 patients and randomly allocated 153 to the placebo group and 151 to the dexamethasone group. 143 (47%) of 304 enrolled patients had pneumonia of pneumonia severity index class 4–5 (79 [52%] patients in the dexamethasone group and 64 [42%] controls). Median length of stay was 6.5 days (IQR 5.0 – 9.0) in the dexamethasone group compared with 7.5 days (5.3 – 11.5) in the placebo group (95% CI of difference in medians 0–2 days; p=0.0480). In-hospital mortality and severe adverse events were infrequent and rates did not differ between groups, although 67 (44%) of 151 patients in the dexamethasone group had hyperglycaemia compared with 35 (23%) of 153 controls (p<0.0001).

### Conclusion

Dexamethasone can reduce length of hospital stay when added to antibiotic treatment in non-immunocompromised patients with community-acquired pneumonia.

## Introduction

The mainstays of treatment for community-acquired pneumonia are early diagnosis and initiation of appropriate antibiotic therapy.[1] Despite preventive measures such as vaccination and advances in antibiotic treatments, community-acquired pneumonia has a high rate of mortality and morbidity and is associated with significant health-care costs.[2]

Adjunctive therapy for community-acquired pneumonia might help to reduce disease severity. In community-acquired pneumonia, locally produced pulmonary cytokines are needed to control and eliminate the primary infection. However, organ dysfunction can result from a systemic inflammatory response.[3] Therefore, a balanced cytokine response needs to be sufficient to control the local infection and not be excessive to prevent systemic effects. An ideal intervention would reduce the systemic complications of the inflammatory response without affecting the resolution of local inflammation.

Corticosteroids are very potent inhibitors of inflammation.[4] They switch off genes that encode proinflammatory cytokines and switch on genes that encode anti-inflammatory cytokines. Treatment with low dose corticosteroids downregulates proinflammatory cytokine transcription, which prevents an extended cytokine response and might accelerate the resolution of systemic and pulmonary inflammation in the early phase of community-acquired pneumonia.[5,6]

Although not all studies show a beneficial effect of corticosteroids, these hormones are widely given as adjunctive therapy in patients with sepsis and septic shock.[7] By contrast with the large number of studies about sepsis and septic shock, there are few controlled trials of corticosteroids as adjunctive treatment to antibiotics in pneumonia, and these trials have produced variable results.[8-10]

We postulated that adjunctive treatment of community-acquired pneumonia with intravenous dexamethasone might change the immune response and thereby reduce morbidity, leading to a decrease in patients' length of stay in hospital. Dexamethasone has potent anti-inflammatory effects and weak mineralocorticoid effects compared with other corticosteroids, thus avoiding interference with sodium reabsorption and water balance. Moreover, dexamethasone has a long-lasting effect, allowing for a once-a-day regimen. We aimed to assess the effect of intravenous dexamethasone compared with placebo on length of hospital stay in non-immunocompromised patients who were admitted to hospital with community-acquired pneumonia.

## Methods

### Study design and patients

We undertook a randomised, double-blinded, placebo controlled trial at the 880-bed St Antonius Hospital in Nieuwegein and the 500-bed Gelderse Vallei Hospital in Ede in the Netherlands (both teaching hospitals). Patients were prospectively enrolled if they were aged 18 years or older and had confirmed community-acquired pneumonia. Diagnosis of pneumonia was confirmed when a new pulmonary infiltrate on a chest radiograph was present in combination with at least two of the following criteria: cough, sputum production, temperature more than 38°C or lower than 35°C, auscultatory findings consistent with pneumonia, C-reactive protein concentration of more than 15 mg/L, white blood cell count of more than $10\times10^9$ cells per L or fewer than $4\times10^9$ cells per L, or more than 10% of rods in leukocyte differentiation.[11] Patients were excluded if they had a known congenital or acquired immunodeficiency or receipt of chemotherapy, any dose of oral corticosteroids, or immunosuppressive medication in the previous 6 weeks) or haematological malignant disease. Patients who needed immediate admission to the intensive-care unit at presentation and pregnant or breastfeeding women were also excluded. Furthermore, patients were not eligible when pneumonia was diagnosed more than 24 h after admission or when the patient needed corticosteroid treatment. Eligible patients provided written informed consent and the study was approved by the institutional Medical Ethics Committee of the St. Antonius Hospital.

### Randomisation and masking

Eligible patients were randomly allocated to receive dexamethasone or placebo by the Department of Clinical Pharmacy (St. Antonius Hospital) in blocks of 20 according to a computer-generated random-number table. Randomisation was based on a one-to-one allocation of prenumbered boxes containing four ampoules (identical appearance for dexamethasone and placebo) for intravenous administration. Patients, investigators, and data assessors were masked to treatment allocation.

### Procedures

Patients in the dexamethasone group were given a bolus of 5 mg (1 mL) of dexamethasone (dexamethasonedisodiumphosphate 5 mg, Centrafarm BV, Etten-Leur, the Netherlands) intravenously and patients in the placebo group were given 1 mL of sterile water for injection (Centrafarm BV) intravenously at the emergency unit, within a maximum of 12 h from admission. All patients received antibiotics before study treatment was given.

For the subsequent 3 days, patients received either intravenous dexamethasone 5 mg (1 mL) or sterile water (1 mL) once a day.

Selection, duration, and administration of the antibiotic treatment were decided by the medical team in charge and were done according to national guidelines.[12] The decision to transfer a patient to the intensive-care unit or hospital discharge were established by their medical team. A general rule for hospital discharge in both hospitals was that patients were clinically stable (improvement of shortness of breath, absence of hyperthermia or hypothermia, consistent decrease of C-reactive protein concentrations, and adequate oral intake and gastrointestinal absorption) and be in a condition to leave the hospital.

The primary endpoint was length of hospital stay in days until hospital discharge or death. If a patient was admitted between 2400 h and 1200 h, the day of admission was counted as 1 day; if the patient was admitted after 1200 h, the day of admission was counted as 0.5 days.

Secondary endpoints included mortality, admissions to intensive-care units, development of empyema, superinfection, readmission, time-courses of C-reactive protein, interleukin-6, and interleukin-10 concentrations, pulmonary function at day 30, and general health-related quality of life as measured by the RAND-36 generic health survey (Appendix A).[13] Pleural effusion was defined as pleural fluid layer thickness on chest radiograph of more than 1 cm that needed additional assessment (ie, pleural puncture), and empyema was defined as pleural effusion containing bacteria. A superinfection was defined as a new infection with or without the need for antibiotic treatment. Readmission was defined as admission to hospital within 30 days from discharge. At a control visit 30 days after the day of admission, lung function was assessed by body plethysmography and carbon monoxide diffusion and helium dilution. Measurements were done in the pulmonary function laboratory of the hospital in which the patient was admitted. Other secondary objectives that were prespecified in the study protocol are beyond the scope of this report and will be reported elsewhere.

We measured concentrations of C-reactive protein with high sensitive-CRP (Roche Diagnostics GmbH, Mannheim, Germany), electrolytes, and glucose, and renal function, liver function, and haematology on the day of presentation. Subsequently, we took samples at 0800 h on days 1–7, if patients were still admitted to the hospital and at a control visit at least 30 days after admission (convalescent phase). We measured interleukin-6 and interleukin-10 concentrations by Milliplex multianalyte profiling (Millipore, Billerica, MA, USA) on the day of presentation and days 1, 2, and 4, and at the control visit. At admission, we measured total serum cortisol concentrations in blood drawn

before administration of the study medication with an ELISA kit (Calbiotech, Spring Valley, CA, USA). Appendix B describes the method used for pathogen identification. Treating doctors assessed comorbidities (neoplastic disease, liver disease, congestive heart failure, renal disease, diabetes mellitus, and chronic obstructive pulmonary disease [COPD]). We calculated a pneumonia severity index score for all patients.[14]

**Statistical analyses**

We calculated the sample size on the basis of the assumption that dexamethasone could reduce the overall length of stay by 2 days. With a reference length of stay of 10 days, we calculated that 150 patients were needed in each group to detect this difference with a power of 80% and a type 1 error of 5% (two-sided). We show number (%) for categorical variables and median (IQR) for continuous variables with non-normal distribution or mean (SD) for those with normal distribution. We assessed differences in categorical variables with the Chi-square test or Fisher's exact test. We analysed differences in length of stay until hospital discharge or death with the Mann-Whitney U test. We calculated 95% CI for differences in medians with an exact test.[15] We also assessed differences in length of stay between treatment groups with the Kaplan-Meier method and a Cox proportional hazard regression model. In these analyses, we made adjustments because patients who died early or were admitted to intensive-care units would count as having a short length of hospital stay. If more patients in the dexamethasone group died after a short length of stay than did in the control group, an incorrect estimate of length of stay would be reported. Equally, patients admitted to the intensive-care unit were all treated with corticosteroids and study medication was stopped after intensive care unit admission. Therefore, we performed a Kaplan-Meier method for analysis of time to discharge, in which patients who were admitted to the intensive-care unit or died were censored to show that the time of reporting was cut-off before the only event of interest for the primary analysis (i.e., hospital discharge) occurred. For the Kaplan-Meier method, a Gehan-Breslow-Wilcoxon test was applied because this test emphasises early differences.[16] In the Cox proportional hazard regression model, we adjusted for all baseline characteristics. To examine differences in quality-of-life scores between the two groups, we calculated the proportion of patients with clinically meaningful changes in quality of life (i.e., a change of ±10 points; Appendix A) between baseline and 1 month after treatment. Differences between the two treatment groups were analysed with the Chi-square test. All statistical analyses were done with SPSS version 15.0. A two-tailed p-value of less than 0.05 was regarded as significant, apart from for multiple comparisons of the quality of life items, in which we used a conservative

value of p<0.01. Interim analyses were preplanned and done after the inclusion of 100 and 200 patients to assess the frequency of serious side effects related to either dexamethasone or placebo. An external, independent data and safety monitoring board reviewed the results of these interim analyses.

## Results

From November, 2007, to September, 2010, we enrolled 304 patients (*Figure 1*, *Table 1*). 133 (44%) patients had comorbidities, with more patients having renal disease in the dexamethasone group than in the control group (*Table 1*). 79 (52%) of 151 patients in the dexamethasone group were in pneumonia severity index risk classes 4 and 5 compared with 64 (42%) of 153 in the placebo group (*Table 1*). Baseline characteristics of patients did not differ between the two hospitals (data not shown).

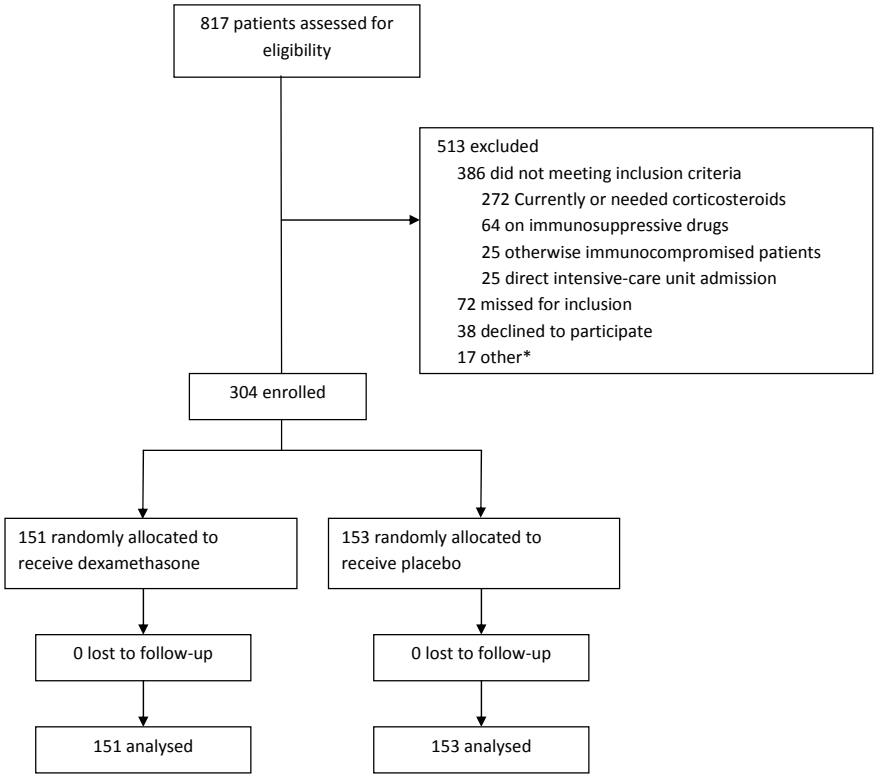



**Figure 1.** Study profile
* Eg, pregnant or breastfeeding.

**Table 1.** Baseline characteristics of enrolled patients

| | **Dexamethasone Group n=151** | **Placebo Group n=153** |
|---|---|---|
| **Sex, male** | 84 (56) | 87 (57) |
| **Age, years** | 64.5 (18.7) | 62.8 (18.2) |
| **Race*** | | |
| Caucasian | 149 (99) | 150 (98) |
| Other | 2 (1) | 3 (2) |
| **Nursing home resident** | 9 (6) | 7 (5) |
| **Current smoker** | 38 (25) | 38 (25) |
| **Antibiotic treatment before admission** | 42 (28) | 39 (25) |
| **Comorbidities†** | | |
| Neoplastic disease | 9 (6) | 10 (7) |
| Liver disease | 2 (1) | 0 |
| Congestive heart failure | 24 (16) | 24 (16) |
| Renal disease | 20 (13) | 10 (7) |
| Diabetes mellitus | 22 (15) | 21 (14) |
| COPD | 20 (13) | 14 (9) |
| **Physical examination findings** | | |
| Temperature (˚C) | 38.2 (1.1) | 38.2 (1.2) |
| Systemic blood pressure (mm Hg) | 130.9 (22.7) | 132.3 (20.7) |
| Heart rate (beats per min) | 96.5 (19.4) | 97.0 (20.2) |
| Respiratory rate (breaths per min) | 24.1 (6.5) | 24.1 (6.7) |
| Altered mental status‡ | 29 (19) | 22 (14) |
| **Laboratory parameters** | | |
| C-reactive protein(mg/l) | 224.5 (143.6) | 209.6 (136.7) |
| White blood count (x $10^9$/L) | 14.7 (6.4) | 14.0 (6.5) |
| Total serum cortisol (μg/dl) | 23.6 (14.9-41.2) | 21.6 (13.5-39.2) |
| **Pneumonia Severity Index Score** | 100.2 (33.4) | 95.8 (32.5) |
| **Pneumonia Severity Index Risk Class,** | | |
| Class 1 | 18 (12) | 22 (14) |
| Class 2 | 30 (20) | 34 (22) |
| Class 3 | 24 (16) | 33 (22) |
| Class 4 | 54 (36) | 43 (28) |
| Class 5 | 25 (17) | 21 (14) |

Data are number (%),mean (SD), or median (IQR).
* self reported. † Patients could have more than one comorbidity. ‡ Defined as a state of awareness that differed from the normal awareness of a conscious person, including sudden confusion, disorientation, or stupor, and scored by the treating doctor.

For the primary outcome, the median length of hospital stay in the dexamethasone group was 6.5 days (IQR 5.0 – 9.0) compared with 7.5 days (5.3 – 11.5) in the placebo group (95% CI of difference in medians: 0–2 days, p=0.0480; *Table 2*). Length of hospital stay differed significantly between groups on Kaplan-Meier analysis (p=0.0478; *Figure 2*). Adjusted for baseline characteristics, the hazard ratio for discharge was 1.46 (95% CI 1.13 – 1.89) favouring earlier discharge for dexamethasone treated patients compared with controls.

**Table 2.** Outcome for all enrolled patients

| | **Dexamethasone Group n=151** | **Placebo Group n=153** | **p Value** |
|---|---|---|---|
| **Length of stay (days)** | 6.5 (5.0-9.0) | 7.5 (5.25-11.5) | 0.0480 |
| **In-hospital mortality** | 8 (5) | 8 (5) | 0.98 |
| Time to death (days) | 5.5 (2.6-18.9) | 8.8 (3.8-12.8) | 0.64 |
| **30 day-mortality** | 9 (6) | 11 (7) | 0.68 |
| **ICU admission** | 7 (5) | 10 (7) | 0.47 |
| Time to ICU admission (days) | 2.5 (1.5-6.5) | 1.8 (1.5-2.6) | 0.34 |
| Length of stay in ICU (days) | 21.5 (14.5-28.5) | 15.5 (10.1-28.5) | 0.23 |
| **Empyema or pleural effusion** | 7 (5) | 5 (3) | 0.54 |
| **Readmission within 30 days from hospital discharge** | 7 (5) | 7 (5) | 0.98 |

Data are median (IQR) or number (%), unless otherwise stated. ICU=intensive-care unit.

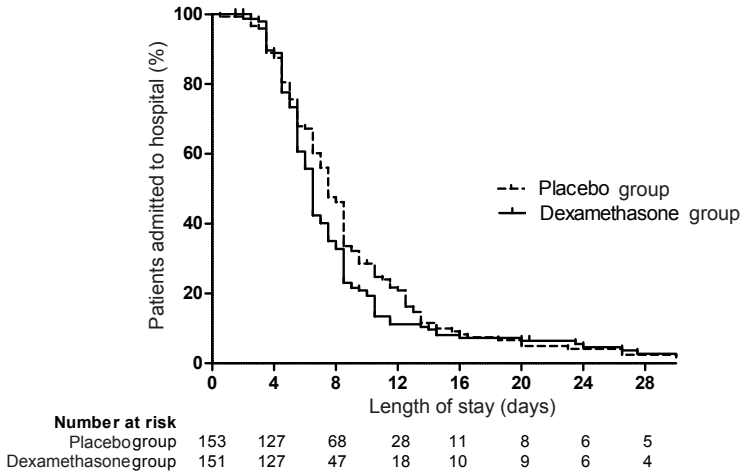
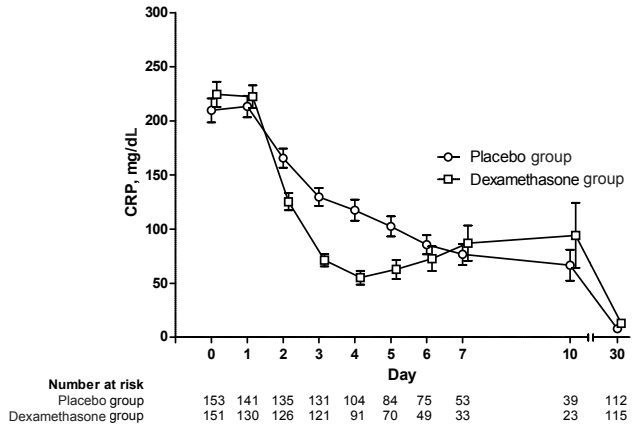


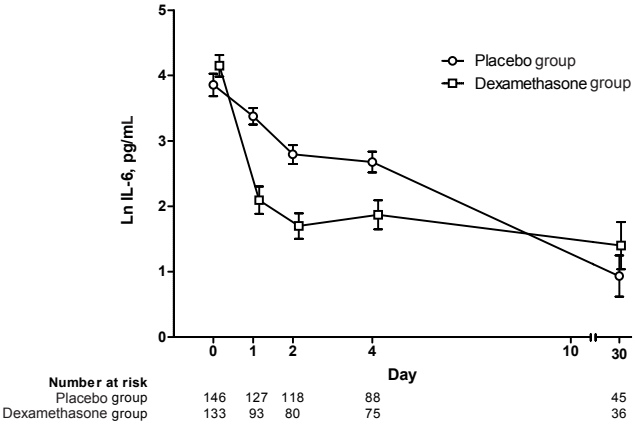

**Figure 2.** Kaplan-Meier analysis of the effect of dexamethasone on length of hospital stay in all enrolled patients. Patients who died or were admitted to the intensive-care unit were censored on the day of death or the day of admission to the intensive-care unit.

All patients were treated with intravenous antibiotics within 4 h of admission to hospital according to national guidelines.[17] Antibiotic treatment was much the same in both groups (Appendix C).[18] (12%) of 151 patients in the dexamethasone group and 16 (10%) of 153 patients in the placebo group were treated with a macrolide alone or as part of combination therapy. Antibiotic treatment was modified on the basis on the outcome of the microbiological investigation. The mean time of switching to oral administration of antibiotics was 5.0 days (SD 4.2) in the dexamethasone group and 5.1 days (3.5) in the placebo group. We established the microbial cause of community-acquired pneumonia in 168 (55%) of 304 patients (Appendix D). *Streptococcus pneumoniae*, *Coxiella burnetii*, *Chlamydophila* spp, and *Legionella* spp were the most frequently identified microorganisms. Distribution of the pathogens did not differ between groups. We noted mixed infection in 21 (7%) patients. 132 (87%) of 151 patients in the dexamethasone group and 134 (88%) of 153 patients in the placebo group completed the 4-day course of study treatment. 13 patients did not complete the course because of admission to intensive-care units, four died, and 21 had protocol violations (Appendix E). For secondary outcomes, hospital mortality (Appendix F) and rates of admission to intensive care units did not differ between groups (*Table 2*). None of the patients received continuous positive airway pressure or non-invasive ventilation outside the intensive care unit. Rates of pleural effusion or empyema were less than 5% in both groups and did not differ significantly (p=0.54; *Table 2*). Seven (5%) patients in both groups were readmitted within 30 days of hospital discharge (Appendix G).

In the first 4 days after admission, we noted a greater decline in C-reactive protein and interleukin-6 concentrations in the dexamethasone group than we did in the control group (*Figure 3*). For interleukin-10, the decrease was much the same between treatment groups. The sharp decrease we noted for interleukin-6 and interleukin-10 concentrations contrasts with the more blunted kinetics of C-reactive protein. On day 10, C-reactive protein concentrations were slightly higher in the dexamethasone group than they were in the placebo group (*Figure 3*).

Concentrations of cortisol before the start of study treatments were much the same between groups. We noted a cortisol concentration of 10 µg/dL or lower in 30 (10%) patients, including 18 (12%) of 149 patients who were tested in the placebo group and 12 (9%) of 141 patients who were tested in



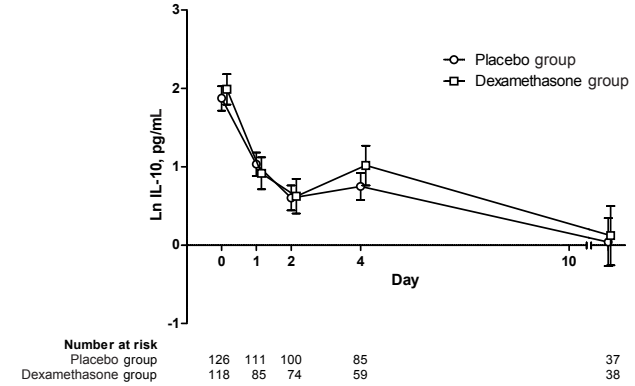




**Figure 3.** Mean concentrations of serum C-reactive protein (A), interleukin-6 (B), and interleukin-10 (C) from hospital admission to day 30. Error bars show standard error. Interleukin concentrations were not tested for all enrolled patients.

the dexamethasone group. In patients with low cortisol concentrations (<10 µg/dL), mortality, intensive-care unit admission, and length of stay did not differ between treatment groups. We assessed lung function at a control visit on day 30 in 93 (61%) patients in the placebo group and 86 (57%) in the dexamethasone group. There were no differences in forced expiratory volume in 1 s (FEV1), FEV1/forced vital capacity, or diffusing capacity of the lung for carbon monoxide in either group (data not shown). 209 (69%) patients completed the RAND-36 quality of life survey on day 3 (114 controls and 95 patients in the dexamethasone group) and 157 (52%) patients completed it on day 30 (79 and 78). Although patients had a similar quality of life on day 3, patients in the dexamethasone group had significant improvements in social functioning by day 30 compared with controls (p=0.0091).

Hyperglycaemia (non-fasting glucose >11 mmol/L[18]) was more common in the dexamethasone group (67 [44%] patients) than it was in controls (35 [23%]; p<0.0001). However, only seven patients (5%) in the dexamethasone group and five patients (3%) in the placebo group needed additional glucose-lowering treatment during their hospital stay (p=0.57). Superinfection occurred in seven (5%) patients in the dexamethasone group and five (3%) patients in the placebo group (p=0.54). One patient in the dexamethasone group had a history of myelodysplastic syndrome, progressed to acute myeloid leukaemia on day 12 after admission, and subsequently died. Another patient in the dexamethasone group had a gastric perforation on day 3. Surgical closure of the perforation was done, and the patient recovered well. Two patients in the placebo group developed an acute myocardial infarction on day 1; one died 4 days after admission to the intensive-care unit and the other patient died after 3 weeks while on the ward. One patient in the placebo group required admission to the cardiac care unit because of new-onset atrial fibrillations. A masked independent monitoring committee (the Medical Ethics Committee, according to predefined regulations) adjudicated all adverse events and decided that there were no reasons for unmasking. Immunological and endocrinological data will be reported elsewhere.

## Discussion

In our trial, we noted an overall reduction in median length of hospital stay of 1 day in patients with community-acquired pneumonia who were given intravenous dexamethasone compared with controls. In a secondary analysis, patients in the dexamethasone group had a better quality of life than did controls with respect to social functioning by day 30 after admission to hospital. These findings support our hypothesis that early administration of dexamethasone changes the immune response and thereby reduces length of

hospital stay in patients with community-acquired pneumonia. This modulation is shown in the accelerated return to normal concentrations of C-reactive protein and interleukin-6 that we noted in the dexamethasone group. However, interleukin-10 concentrations were not affected by the use of dexamethasone. The published effects of glucocorticosteroids on interleukin-10 concentrations during infection are variable,[19-21] and the effect of dexamethasone on interleukin-10 is probably dose-dependent.[22] We reported an apparent rebound effect of dexamethasone on C-reactive protein concentrations by day 10 after admission to hospital, as previous described in the published work.[23] However, this finding might be explained because, by day 10, most patients in the dexamethasone group had been discharged, whereas the remaining patients had a complicated clinical course. By contrast, on day 10 the placebo group had a high number of patients who were almost ready for discharge, and had low mean C-reactive protein concentrations. Moreover, the number of readmissions was not higher in the dexamethasone group than the control group, which would have been expected in the case of a true rebound effect.

Our results are in line with other studies that showed a beneficial effect of corticosteroids in patients with community-acquired pneumonia. Confalonieri and colleagues[24] reported an improvement in oxygenation and a survival advantage in patients with severe community-acquired pneumonia who were treated with hydrocortisone for 7 days. A retrospective study[25] suggested that patients with severe community-acquired pneumonia who were treated with systemic corticosteroids had a reduced risk of mortality compared with patients who were not given adjunctive corticosteroids. A small randomised-controlled trial[26] of 31 patients with community-acquired pneumonia of any severity compared prednisolone for 3 days with placebo and reported a non-significant reduction in hospital stay from 16 to 11 days (p=0.182). However, this study was probably too small to show significant effects on length of stay. A study[27] of 213 patients—the largest so far to assess the role of prednisolone (40 mg once per day for 7 days) in community-acquired pneumonia of any severity—showed neither beneficial effects of adjunctive corticosteroids on clinical cure at day 7 or effects on length of stay. A possible explanation for the absence of effect compared with our study was the use of prednisolone once a day, which might not have been sufficient to achieve effective serum concentrations during the course of 24 h. Furthermore, this study was not powered to show differences in the length of hospital stay.

In our study, the median length of hospital stay of 7.5 days in the placebo group was reduced by one day by dexamethasone (13% reduction). Although the group size of the study was calculated for a 2 day reduction, we regard the noted 1 day reduction as clinically relevant.

Our study has several strengths compared with previous studies. It was the largest randomised double-blind, placebo-controlled trial undertaken to date and was done in two hospitals. We used dexamethasone, which has a comparatively long biological half-life of 36–54 h.[28] Because we provided dexamethasone once a day for 4 days, the pharmacological effects can be expected from day 1 to about day 11. Moreover, because of the long half-life of dexamethasone, a more gradual reduction in biological effects might be expected, allowing for a gradual increase in intracellular glucocorticoid receptor number and recovery of the hypothalamic-pituitary-adrenal axis. Additionally, we measured total cortisol concentrations on the day of admission to detect adrenal insufficiency. The significance of a low serum cortisol concentration in patients with community-acquired pneumonia is, however, not clear.[29] Nevertheless, in accordance with other studies, total cortisol concentrations of lower than 10 μg/dL were not associated with worse outcome than were higher concetrations.[30] Pneumonia severity index risk classes 4 and 5 were more commonly noted in the dexamethasone group than the placebo group. This imbalance in the severity of community-acquired pneumonia could have led to an underestimation of the effect of dexamethasone because a high risk class (4 or 5) usually leads to a longer length of stay than does a low risk class (1–3).[31] Our study had limitations. First, the results cannot be generalised to all patients with community-acquired pneumonia. In patients with COPD, pneumonia is usually coincident with bronchial obstruction, which needs treatment with systemic corticosteroids[32] and therefore led to an underrepresentation of patients with COPD in this study (only 34 [11%] of 304 patients enrolled this study had COPD compared with an incidence of around 21% of the 817 people in the screened population). Also, the microorganism *C. burnetii* is somewhat overrepresented in this study because of an outbreak of Q fever in the Netherlands in spring 2009.[33] However, patients with *C. burnetii* pneumonia were equally distributed between the dexamethasone and placebo groups. Another limitation was that, because of low rates of antibiotic resistance, guidelines for antibiotic treatment in the Netherlands differ from US guidelines.[34] In the Netherlands, amoxicillin is standard therapy for community-acquired pneumonia of pneumonia severity index class 1 and 2 and is combined with a fluoroquinolone or macrolide antibiotic in patients with more severe community-acquired pneumonia. All pneumococci derived from sputum or blood cultures in this study were sensitive to penicillin. A further limitation was that admission to intensive-care units during the hospital stay was defined as an endpoint of this study. Patients with severe community-acquired pneumonia who were admitted to the intensive-care unit were given corticosteroids according to the Surviving Sepsis Campaign

protocol.[35] Therefore, we were unable to assess the effects of dexamethasone on mechanically ventilated patients. The study was not sufficiently powered to show an effect of dexamethasone on admission to the intensive-care unit. Finally, dexamethasone was given intravenously. Although the study protocol allowed health-care professionals to stop the intravenous administration of dexamethasone if patients were switched to oral antibiotics, most patients received the full course of study medication. Therefore, participation in the trial might have resulted in longer administration of intravenous antibiotics. Although serious adverse events were rare, one patient in the dexamethasone group developed a gastric perforation on day 3 that could be attributed to the use of dexamethasone. Furthermore, hyperglycaemia was noted more often in the dexamethasone group than it was in the control group. Hyperglycaemia is also associated with adverse outcome in non-critically ill patients.[36] The benefits of corticosteroids should be weighed against the potential disadvantages of these drugs, such as superinfections and gastric disturbances.

## Appendix A

### Rand-36 assessment

The RAND-36 assesses physical and social functioning, physical and emotional role restriction, mental health, vitality, pain, general health and change in health in the 30 days preceding the assessment. The first questionnaire was taken on day three of admission, and the second one was handed over to the patient 30 days after the day of admission to the hospital, i.e., at the moment of CRP measurement. Scales and items of RAND-36 range in score from 0 to 100. A high score represents a high level of quality of life and better functioning. A change of 10% of the scale breadth is perceptible to patients as a meaningful change, and a change in quality of life of 10 points is therefore considered clinically relevant.[37]

## Appendix B

### Pathogen identification

At least two sets of separate blood samples (drawn before the start of in-hospital antibiotic treatment) from each patient were cultured. Sputum specimens (if available) were Gram-stained and cultured; sputum samples with positive cultures were only used for further analysis if they fulfilled our definitions of representative sputum.[38] Urine antigen tests were performed for the detection of *Legionella pneumophila* serogroup 1 and *Streptococcus pneumoniae* (enzyme immunoassay, Binax-NOW; Binax, Portland, ME, USA). In-house developed polymerase chain reactions (PCR, Taqman real-time) were performed on the sputum to detect *Legionella* spp., *Mycoplasma pneumoniae*, *Coxiella burnetii*, and *Chlamydophila psittaci*.[39] Paired serological testing was performed for the presence of antibodies to *Mycoplasma pneumoniae*, *Coxiella burnetii*, *Chlamydophila* spp. or respiratory viruses (adenovirus, influenza virus A and B, parainfluenza virus 1, 2 and 3 and the respiratory syncytial virus) (Serodia, Bipharma, Fujirebio Inc., Tokyo, Japan). A four-fold increase in antibody titre was considered positive. Pharyngeal samples were taken for viral culture or PCR ((para-) influenza virus, adenovirus, respiratory syncytial virus).

## Appendix C

Antibiotic treatment in the two treatment groups.

| Antibiotics | Dexamethasone group n=151 | Placebo group n=153 |
|---|---|---|
| **Amoxicillin or Amoxicillin clavulanic acid** | 61 (40.4) | 74 (48.4) |
| **Amoxicillin clavulanic acid and macrolide** | 14 (9.3) | 10 (6.5) |
| **Amoxicillin clavulanic acid and fluorquinolone** | 12 (7.9) | 9 (5.9) |
| **Amoxicillin clavulanic acid and oseltamivir** | 0 (0.0) | 1 (0.7) |
| **Cephalosporine** | 43 (28.5) | 40 (26.1) |
| **Cephalosporine and macrolide** | 3 (2.0) | 3 (2.0) |
| **Cephalosporine and fluorquinolone** | 10 (6.6) | 5 (3.3) |
| **Cephalosporine and fluorquinolone and oseltamivir** | 1 (0.7) | 0 (0.0) |
| **Tetracycline** | 2 (1.3) | 5 (23.3) |
| **Fluorquinolone** | 1 (0.7) | 2 (1.3) |
| **Macrolide** | 1 (0.7) | 3 (2.0) |
| **Cotrimoxazole** | 3 (2.0) | 1 (0.7) |

Data are number (%).

## Appendix D

Microbiological causes of pneumonia in the two treatment groups.

| | Dexamethasone Group n=151 | Placebo Group n=153 |
|---|---|---|
| ***Streptococcus pneumoniae*** | 38 (25)[1] | 34 (22)[2] |
| ***Legionella* spp.** | 5 (3.3) | 7 (4.6) |
| ***Chlamydophila* spp.** | 8 (5.3)[3] | 6 (3.9) [4] |
| ***Coxiella burnetii*** | 11 (7.3) | 16 (11)[5] |
| ***Mycoplasma pneumoniae*** | 2 (1.3) | 3 (2.0)[6] |
| ***Staphylococcus aureus*** | 0 (0.0) | 3 (2.0) |
| ***Haemophilus influenzae*** | 3 (2.0)[7] | 6 (3.9)[8] |
| **Other Gram-stain positive bacteria** | 4 (2.6) | 0 (0.0) |
| **Other Gram-stain negative bacteria** | 5 (3.3) | 5 (3.3) |
| **Influenza A/B virus** | 2 (1.3) | 5 (3.3)[9] |
| **Other viruses** | 3 (2.0) | 9 (5.9) |
| **Unidentified** | 70 (46) | 59 (39) |

Data are number (%)
Gram-stain positive: *Rhodococcus equi*, Group A streptococci, Group G streptococci. Gram-stain negative: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Serratia marcescens, Enterobacter cloacae*
Other viruses: adenovirus, parainfluenzavirus, respiratory syncytial virus, rhinovirus.
[1]: Mixed infection with influenza virus (n=4) or parainfluenza virus (n=3) or hMPV (n=1). [2]: Mixed infection with influenza virus (n=2) or parainfluenza virus (n=1). [3]: Mixed infection with *H. influenzae* (n=1). [4]: Mixed infection with parainfluenza virus (n=1). [5]: Mixed infection with influenza virus (n=2) or parainfluenza virus (n=1). [6]: Mixed infection with rhinovirus and parainfluenza virus (n=1). [7]: Mixed infection with influenza virus (n=1) or parainfluenza virus (n=1). [8]: Mixed infection with *S. pneumoniae* (n=1). [9]: Mixed infection with RS virus (n=1)

## Appendix E

**Reasons for protocol violation.**

Of the 21 patients, in five patients in the dexamethasone group and three patients in the placebo group, the attending physician overruled the study protocol and started corticosteroids. The reason for overruling was COPD in five patients, concurrent extrinsic allergic alveolitis in one patient and severe dyspnoea in two patients. Two patients (one in each group) received one injection of hydrocortisone 100 mg for hypotension on day two. In the dexamethasone group, two patients needed an additional course of corticosteroids after they stopped receiving the study medication, compared to six patients in the placebo group. In most patients, the reason for this additional therapy was persistent wheezing, probably due to an exacerbation of COPD.

## Appendix F

Information on patients who died.

| Patients | Age (year) | PSI score | Cause of death |
|---|---|---|---|
| **Placebo** | | | |
| 1 | 72 | 122 | Sepsis, respiratory failure |
| 2 | 92 | 172 | Sepsis, respiratory failure |
| 3 | 60 | 90 | Spondylodiscitis, multi organ failure. |
| 4 | 57 | 117 | Severe *Legionella* pneumonia, respiratory failure. |
| 5 | 89 | 139 | Myocardial infarction |
| 6 | 77 | 87 | Respiratory failure in recovery phase after aspiration. |
| 7 | 79 | 169 | Myocardial infarction |
| 8 | 78 | 178 | Sepsis, respiratory failure |
| **Dexamethasone** | | | |
| 1 | 95 | 185 | Sepsis, respiratory failure |
| 2 | 91 | 141 | Cardiac arrest. |
| 3 | 93 | 143 | Cardiac arrest. |
| 4 | 86 | 106 | Sepsis, respiratory failure |
| 5 | 81 | 71 | Progressive heart failure |
| 6 | 89 | 149 | Known myelodysplastic syndrome progressed to acute myeloid leukaemia in recovery phase |
| 7 | 68 | 148 | Sepsis, respiratory failure |
| 8 | 82 | 132 | Sepsis, respiratory failure |

## Appendix G

### Reasons for readmission

In the dexamethasone group, six patients were readmitted for pulmonary manifestations (relapse of pulmonary infection (5), empyema (1)), and one patient was readmitted for dehydration. In the placebo group, five patients were readmitted for pulmonary manifestations (relapse of pulmonary infection (3), haematothorax (1) and pleural pain (1)), and two patients were readmitted for non-pulmonary indications (urosepsis (1) or diarrhoea with dehydration (1)).

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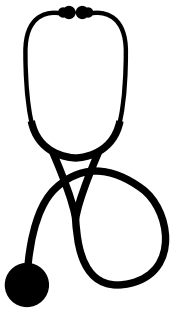

# Chapter 11

# Community-acquired pneumonia: Cytokine response may predict benefit from dexamethasone

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## Abstract

### Background

Corticosteroids can improve the clinical outcome in patients with pneumonia, however, little is known about the effect on systemic cytokine levels. Furthermore, it is not known which patients benefit most from corticosteroid therapy.

### Methods

304 hospitalised, non-immunocompromised patients with community-acquired pneumonia (CAP) were randomly assigned to receive either an adjunctive four-day course of dexamethasone (5 mg once a day) or placebo. Serum levels of IL-1Ra, IL-6, IL-8, IL-10, IL-17, TNF-α, INF-γ, MIP and MCP were measured at various time-points during hospital stay.

### Results

304 Patients (153 placebo and 151 dexamethasone) were analysed. The median levels of IL-6 ($p<0.001$), MCP ($p<0.001$), TNF-α ($p<0.001$), IL-8 ($p=0.001$) and IL1-Ra ($p=0.098$) were significantly lower on day 2 in the dexamethasone treated patients compared to the placebo group. Patients with IL-6, IL-8 and MCP above predefined cut-off levels benefited most from dexamethasone therapy. In the dexamethasone group only 2/24 patients (8.3%) died or were admitted to the intensive care unit (ICU), while this occurred in 8/17 patients (47%) of the placebo group ($p<0.01$).

### Conclusion

Cytokine and chemokine levels decreased more rapidly in the dexamethasone treated patients compared to the placebo group. Especially, in patients with a high cytokine response, dexamethasone showed a reduction in mortality and ICU admittance.

## Introduction

Glucocorticosteroids are physiological inhibitors of the inflammatory response and are widely used as adjunctive treatment in various inflammatory diseases, such as meningitis and sepsis.[1-4] In the last decades, there have been major advances in understanding the molecular mechanisms by which glucocorticosteroids suppress inflammation. During infection, invading pathogens shed outer membrane components into the environment. As a result, inflammatory cells become activated and start to secrete cytokines and chemokines. These cytokines and chemokines control and eliminate the infection by leukocyte recruitment and inflammation. If not regulated tightly, the innate inflammatory response can result in progression from SIRS to sepsis and septic shock and ultimately multiple organ dysfunction syndrome (MODS). The binding of corticosteroids to their receptors in the cytoplasma eventually results in changes of gene transcription and lead to suppression or, sometimes, upregulation of the inflammatory cytokines.[5-7]

Prolonged administration of low-dose glucocorticosteroids can improve clinical outcome in patients with septic shock.[8,9] Despite this beneficial effect, relative little research has been performed on the clinical effects of corticosteroids on patients with milder infections such as pneumonia, as well as the cytokine response during this disease. In the available literature on the effect of corticosteroids on cytokine levels in community-acquired pneumonia (CAP), no distinction was made between the effect of dexamethasone in patients with a high or low cytokine response.[10-15]

In this study we evaluated the effect of dexamethasone on the cytokine response in patients with CAP. We explored which patients benefited most from dexamethasone therapy during CAP.

## Methods

### Patients

The analyses were based on all patients with CAP enrolled in a study on the effect of dexamethasone on length of hospital stay.[16] From November 2007 until September 2010, patients with confirmed pneumonia were prospectively enrolled in this study at the St. Antonius Hospital in Nieuwegein or at the Gelderse Vallei Hospital in Ede, both teaching hospitals (600 respectively 500 beds) in the Netherlands. The diagnosis pneumonia was made when the chest X-ray showed an infiltrate in combination with at least two of the following criteria: cough, sputum production, temperature >38˚C or <35˚C, auscultatory findings consistent with pneumonia, C-reactive protein (CRP) >15 mg/l, or leukocytosis or leukopenia (white blood count >10x10$^9$/L, <4x10$^9$/L,

respectively, or >10% rods in leukocyte differentiation).[17] Patients with defined immunodeficiencies (a known congenital or acquired immunodeficiency, chemotherapy within the last 6 weeks, any use of oral corticosteroids use in the last 6 weeks, immunosuppressive medication in the last 6 weeks), haematological malignancies, pregnancy, or breast feeding were excluded. Moreover, patients were excluded when pneumonia was diagnosed >24 hours after admission or when the patient required corticosteroid use. Mortality, intensive care unit (ICU) admission, length of hospital stay (primary endpoint of the study), and the causative microorganism were assessed. Pathogen identification is described in the appendix A.

**Study design**

Patients were randomised to receive a bolus of 5 mg (1 mL) of dexamethasone (dexamethasonedisodiumphosphate 5 mg, Centrafarm BV, Etten-Leur, the Netherlands) IV or 1 mL of sterile water (water for injection, Centrafarm BV, Etten-Leur, the Netherlands) IV on the emergency unit. On the following three days the patient received either intravenously dexamethasone 5 mg (1 mL) once a day or sterile water 1 mL once a day. Randomisation was based on a one-to-one allocation by means of pre-numbered boxes containing four ampoules for IV administration. Patients, investigators, and those assessing the data were masked to allocation. The local Ethics Committee approved the study and informed consent was obtained from all participants.

**Determination of the cytokine response**

Systemic circulating concentrations of interleukin-1 receptor antagonist (IL-1Ra), interleukin-6 (IL-6), IL-8, IL-10, IL-17, tumour necrosis factor-α (TNF-α), interferon-γ (INF-γ), macrophage inflammatory protein (MIP) and monocyte chemotactic protein (MCP) were measured on day of presentation (before the first administration of dexamethasone) by Milliplex multi-analyte profiling (Millipore, Billerica, MA, USA) and subsequent samples were drawn at 8 A.M. on days 1, 2, 4, and at a control visit at least 30 days after admission (convalescent phase). To analyse whether these cytokines and chemokines act as acute phase proteins, we analysed the difference in cytokine levels between the acute and convalescent phases. An acute phase response was defined as a decrease or increase in the cytokine or chemokine level by at least 25% in the acute phase compared to the convalescent phase.[18]

**Statistical analyses**

All statistical analyses were performed using statistics software (SPSS version 15.0 for Windows, Chicago, IL, USA). A two-tailed p-value of <0.05 was considered significant.

Differences in continuous variables were tested by the Student's t test or the Mann-Whitney U test, as appropriate. When linear regression analysis was used, we included cytokine concentration on day 0 (independent variable) to predict cytokine concentration on day 2 (dependent variable). Because the cytokines were not normally distributed, except for CRP, the cytokines were transformed in a natural log scale for the linear regression. In the linear regression analysis we chose to analyse the decrease of the cytokines from day 0 to day 2 because all patients using corticosteroids were at least 24 hours on corticosteroids on day 2 and because cytokine concentrations decreased most in the first days. To correct for the magnitude of the cytokine response on day 0, this is an independent variable in the linear regression. In a *post hoc* analysis to predict the patients who will benefit from corticosteroids, we analysed the cytokine levels of patients who deceased or were admitted to the ICU and compared those with  patients who did not (Student's t test). IL-6, IL-8, IL-10 and MCP levels turned out to be significantly higher in the deceased/ICU group. For these cytokines, ROC curve analysis was performed to identify cut-off points with optimal predictive values. For CRP, a ROC analysis showed an AUC of 0.505. Therefore we used the mean CRP level of all patients as cut-off level.

## Results

A total of 304 patients were enrolled in the study; 151 patients received four days of dexamethasone and 153 patients received placebo.[19] The baseline characteristics of the patients are shown in *table 1*. In 175 (57%) patients an aetiological diagnosis of the CAP could be made. In 24% of the patients *S. pneumoniae* was detected, in 19% an atypical bacterium (*Legionella pneumophila*, *Mycoplasma pneumoniae*, *Coxiella burnetii*, or *Chlamydophila psittaci*), in 6% Gram-negative bacteria, in 6% a viral pathogen and in 2% other Gram-positive bacteria were found. There were no significant differences between the dexamethasone and placebo group.

**Table 1.** Baseline characteristics of the 304 patients with CAP receiving dexamethasone or placebo.

| | **Dexamethasone Group n=151** | **Placebo Group n=153** |
|---|---|---|
| **Sex, male** | 84 (57) | 87 (56) |
| **Age, years** | 64.5 (18.7) | 62.8 (18.2) |
| **Race*** | | |
| Caucasian | 149 (99) | 150 (98) |
| Other | 2 (1.3) | 3 (2.0) |
| **Nursing home resident** | 9 (6.0) | 7 (4.6) |
| **Current smoker** | 38 (27) | 38 (27) |
| **Antibiotic treatment before admission** | 42 (28) | 39 (26) |
| **Comorbidities** | | |
| Neoplastic disease | 9 (6.0) | 10 (6.5) |
| Liver disease | 2 (1.3) | 0 (0.0) |
| Congestive heart failure | 24 (16) | 24 (16) |
| Renal disease | 20 (13) | 10 (7) |
| Diabetes mellitus | 22 (15) | 21 (14) |
| COPD | 20 (13) | 14 (9) |
| **Pneumonia Severity Score** | 100.2 (33.4) | 95.8 (32.5) |
| **Pneumonia Severity Risk class** | | |
| Class 1 | 18 (11.9) | 22 (14.4) |
| Class 2 | 30 (19.9) | 34 (22.2) |
| Class 3 | 24 (15.9) | 33 (21.6) |
| Class 4 | 54 (35.8) | 43 (28.1) |
| Class 5 | 25 (16.6) | 21 (13.7) |
| PSI class IV en V | 79 (52.3) | 64 (41.8) |

Data are presented as number (%) or mean (SD)
*: Race was reported by the patient.

## Cytokine and chemokine response at admission

Comparison of the CRP levels at day 0 with the levels at day 30 clearly showed the acute phase response of this protein (+2870% at day 0 as compared to day 30 levels). An acute phase response was also observed for the cytokines IL-6 (+2603 %), IL-1Ra (+700 %), IL-10 (+326%), IL-8 (+178 %) and TNF-α (+104 %). In most patients the cytokine INF-γ and chemokine MIP were undetectable. However, in patients where levels were detectable, INF-γ (+1315 %) showed a positive acute phase response but MIP(+4.4%) did not. The chemokine MCP (+8.0%) also did not show an acute phase response (*Figure 1*).

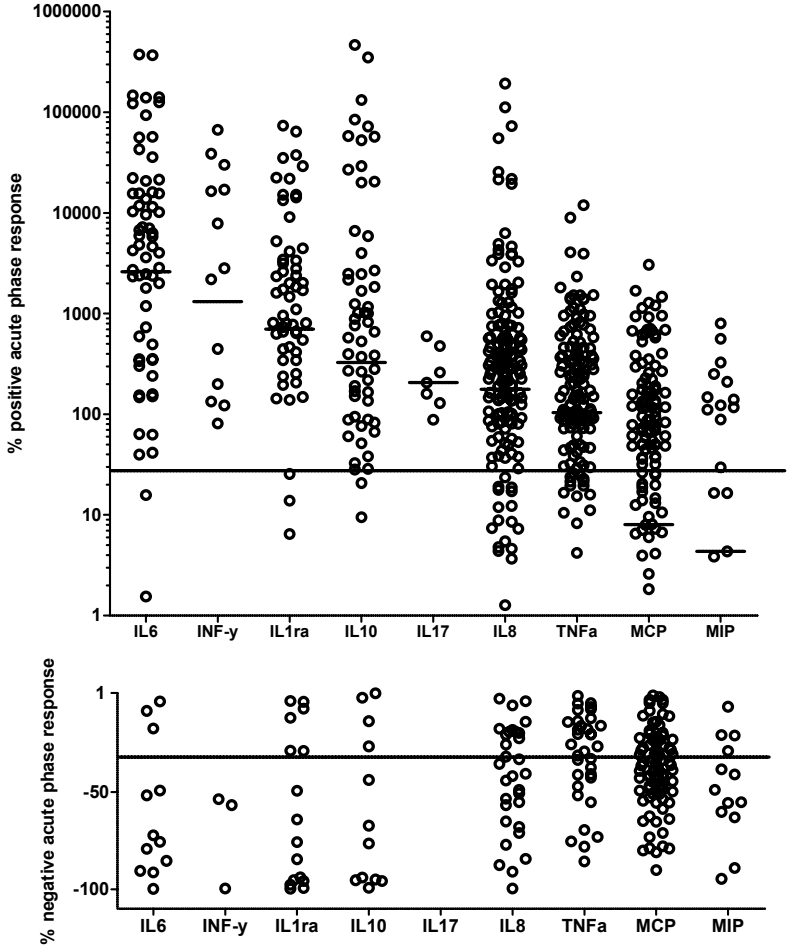


**Figure 1.** Dynamics of the systemic cytokine response during community-acquired pneumonia.

The relative change (%) of the cytokines in the acute phase of disease compared to the convalescent phase is plotted. The cut-off for positive (+25%) and negative (-25%) acute phase responses are plotted (drawn line). Data points of individual patients are shown as open circles; horizontal bars indicate median values. Positive acute phase responses (i.e. increased during inflammation) are indicated in the upper panel on a logarithmic scale; negative acute phase responses (i.e. decreased during inflammation) are indicated in the lower panel on a linear scale.

**Dexamethasone reduces the magnitude of the cytokine and chemokine response.**

The data above show the cytokine response at day 0, in comparison with day 30 of the whole group. However, half of the patients received dexamethasone during hospitalisation. This changed the dynamics of the cytokines response during hospital stay (*Figure 2*). Cytokine levels were similar on the day of admission, but the median levels of IL-6 ($p<0.001$, β: -1.319 (-73%)), MCP ($p<0.001$, β: -0.385 (-32%)), TNF-a ($p<0.001$, β: -0.484 (-38%)), IL-8 ($p=0.001$, β: -0.423 (-35%)) and IL1-Ra ($p= 0.098$, β:-0.431 (-35%)) were significantly lower on day 2 in the dexamethasone treated patients compared to the placebo treated patients. These data indicate that prototype pro-inflammatory cytokines return to normal faster in the dexamethasone treated patients.

CRP also showed a more rapid decrease in the dexamethasone group ($p<0.001$, β: -0.237), but this was more blunted than IL-6. For MIP and IFN-γ only a trend towards a more rapid decrease in the dexamethasone group was seen. IL-10 showed a rapid decrease in the dexamethasone treated group as well as the placebo group.

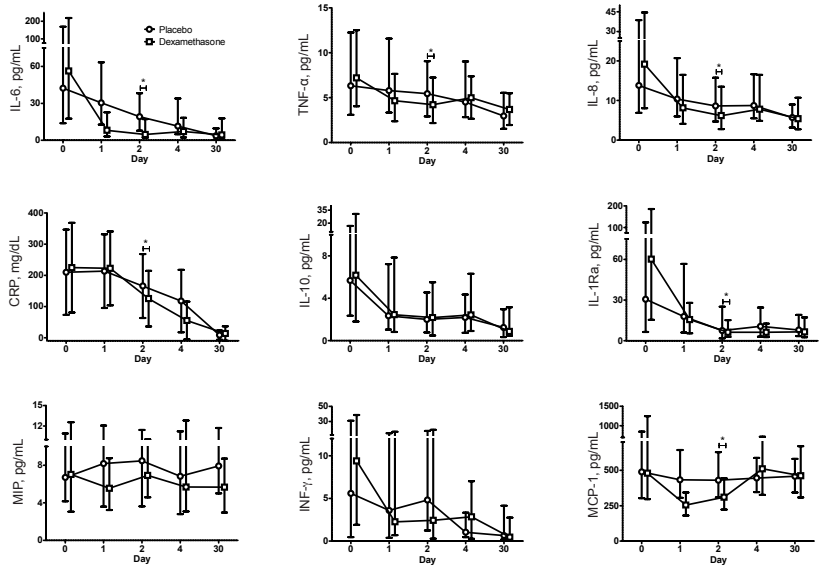


**Figure 2.** The effect of dexamethasone on the cytokine response.
Median plasma cytokine concentrations with the interquartile range on subsequent days in the dexamethasone and placebo group. *: Statistically significant

**Dexamethasone reduces ICU admission and mortality in patients with a high cytokine response.**

In a previous cohort of patients with CAP, patients who died or required admission to the ICU during hospitalisation, IL-6 (p=0.038), IL-8 (p=0.014), IL-10 (p=0.024) and MCP (p=0.001) were significantly higher on the day of admission compared to patients who survived or did not require ICU admission.[20] In our current cohort of patients the same predicting cytokines for these outcome parameters were found, except for IL-6 (p=0.134). In patients who died or were admitted to the ICU during hospitalisation, IL-8 (p<0.01), IL-10 (p=0.030) and MCP (p=0.026) were higher on the day of admission compared to patients who did not. We therefore analysed whether patients with a high cytokine response (and therefore at higher risk for death or ICU admission) would benefit more from dexamethasone treatment than patients with a more modest cytokine response. Because dexamethasone has little or no influence on IL-10 levels, we constructed a model with IL-6, IL-8 and MCP. To that end we selected all patients in which all these three cytokines (IL-6 (≥ 4.53 mg/dL), IL-8 (≥ 3.79 mg/dL) and MCP (≥ 6.70 mg/dL) were elevated. In these patients with an elevated cytokine response, 2 of the 24 patients in the dexamethasone group died (8.3%) compared to 8 out of 17 patients (47%) in the placebo group (p<0.01, OR: 0.10; 95% CI 0.02 – 0.58) (*Figure 3a*). In the patients with a moderate cytokine response (none of the three cytokines were elevated), mortality and ICU admittance did not significantly differ between the dexamethasone and placebo group (dexamethasone 7.5% vs. placebo 3.6%; p=0.468). In the patients in which all three cytokines were elevated, median length of stay was significantly lower in the dexamethasone group compared to the placebo group (6.5 days (IQR 5.5 – 10.3) vs. 12 days (IQR 7.3 – 31); p=0.028)

Although a combination of the three cytokines shows best which patients benefit from dexamethasone, this effect was mainly caused by the IL-8 level upon admission. When only a high IL-8 level (≥3.79 mg/dL) was used, 5 out of 36 patients (14%) died in the dexamethasone group compared to 9 out of 27 patients (33%) in the placebo group (p=0.066).

When CRP was used to predict which patients benefit most from dexamethasone, for patients with a CRP>217 mg/L no more than a trend towards a reduction in mortality or ICU admission in dexamethasone treated patients was seen. (*Figure 3b*). In the patients with a CRP >217 mg/L, median length of stay was significantly lower in the dexamethasone group compared to the placebo group (6.5 days (IQR 4.5 – 9.5) vs. 8.5 days (IQR 5.5 – 13.4); p=0.013).

Above data indicate that the patients with the highest proinflammatory cytokine response benefit most from dexamethasone. This did not hold true

for the severity of CAP as determined by the PSI score: 62% of the patients with a high cytokine response treated with dexamethasone were in PSI class 4 or 5, compared to 55% of the patients with a high cytokine response and placebo. Of the patients who eventually died or went to the ICU this was 67% (dexamethasone) vs. 63% (placebo).

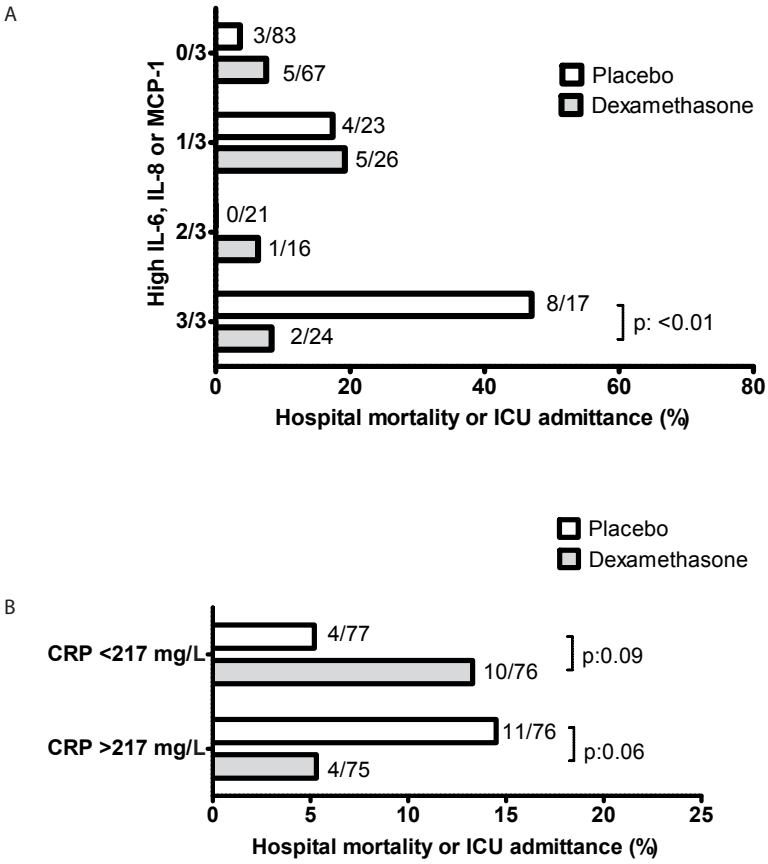


**Figure 3.** The effect of dexamethasone on outcome in relation to cytokine response and CRP levels.
A: The effect of dexamethasone on a combined endpoint of mortality and/or ICU admission in relation to elevated levels of IL-6 (≥4.53 mg/dL), IL-8 (≥3.79 mg/dL) and MCP (≥6.70 mg/dL).
0/3: None of the cytokines was elevated. 1/3: One of the three cytokines was elevated. 2/3: Two of the three cytokines were elevated. 3/3: All three cytokines were elevated.
B: The effect of dexamethasone on a combined endpoint of mortality and/or ICU admission in relation to CRP level.

## Discussion

In this study we have shown that in patients with CAP, cytokine and chemokine levels decrease more rapidly in dexamethasone treated patients compared to the placebo treated patients. In patients with high levels of IL-6, IL-8 and MCP, dexamethasone showed a reduction in a combined endpoint of mortality and ICU admission.

Our results are in concordance with other studies that demonstrated a lowering effect of corticosteroids on most of the cytokine and chemokine levels during infection.[14,21-25] We found no influence of dexamethasone on IL-10 levels. The described effects of glucocorticosteroids on IL-10 levels during infection are variable; IL-10 levels have been reported to increase, to decrease or not to change at all.[26-28] Most probably, the effect of dexamethasone on IL-10 is dose-dependent.[29] Most of the studies on corticosteroids in relation with cytokine response are performed in patients with septic shock.[14,30-32] Only two studies investigated the cytokine response in patients with pneumonia, although in one study all patients were mechanically ventilated, while in the study of Marik *et al*. only patients with severe pneumonia admitted to the ICU were evaluated.[33,34] None of the studies examined if patients with a high cytokine response benefited more from corticosteroid therapy compared to patients with a low cytokine response.

In the overall patient cohort we were able to show an effect of dexamethasone on length of hospital stay, but no effect on patient survival and/or ICU admission.[35] This may be caused by the fact that more patients in the dexamethasone group had a pneumonia ranked as PSI class 4 or 5 and therefore more patients were expected to die. Indeed, when we compared patients with a high cytokine response to all other patients, we did find an effect of dexamethasone on hospital mortality and ICU admittance. IL-6, IL-8, and MCP have been found previously to correlate with disease severity or mortality in patients with CAP.[36-39] Dexamethasone suppresses the response of these cytokines, however, contrary to other corticosteroids, dexamethasone has no direct effect on fluid balance or hemodynamic parameters. This demonstrates that suppression of the immune response only, can result in survival benefit. Most likely, patients with a high cytokine response at admission have more benefit from dexamethasone than patients without. This clear distinction could not be made based on CRP or a single cytokine or a combination of CRP with either IL-6, IL-8 or MCP.

Interestingly, only 59% of the patients with a high cytokine response had a CAP ranked as PSI class 4 or 5. Therefore, not only patients with PSI class 4 or 5 will benefit from corticosteroids. For example, dexamethasone can prevent ICU admission in young patients (with, because of their age, a low PSI class) with severe CAP and thus, a high cytokine response. Suppression of the cytokine

response might not always be beneficial. In the patients with none of the three cytokines elevated, a higher mortality/ICU admission is seen, although this is not statistically significant (3.6 vs. 7.5%; p=0.468).

This study has some limitations. First, analyses on cytokines and CRP as predictive values for the beneficial effect of dexamethasone are *post hoc* and should therefore be interpreted with caution. Second, the selection of the most relevant cytokines for the prediction of the effect of dexamethasone is determined by the most prevalent causative agent, in essence *S. pneumoniae*, an extracellular pathogen. It is conceivable that atypical microorganisms, mostly intracellular pathogens, induce a cytokine response with a different spectrum. The relative small sample size of the latter group does not allow to conclude whether patients with an atypical pathogen benefit from dexamethasone. Third, at the moment real-time measuring of cytokines at the emergency department is not yet available and would be very expensive.

In conclusion, patients with CAP treated with dexamethasone in addition to antibiotic treatment show a more rapid decrease of cytokine levels. A subgroup of patients with a high cytokine response benefitted most from dexamethasone in terms of hospital mortality and ICU admission. A randomised controlled trial of dexamethasone administration in CAP patients with a high cytokine response should be performed to confirm our findings.

## Appendix A

### Pathogen identification

At least two separate blood samples, and sputum samples from each patient were cultured. Urine antigen tests were performed for the detection of *Legionella pneumophila* serogroup 1 (Binax-Now; Binax, Portland, ME, USA) and *Streptococcus pneumoniae* (Binax-Now; Binax, Portland, ME, USA). In-house developed polymerase chain reactions (PCR) were performed on the sputum to detect *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Coxiella burnetii*, and *Chlamydophila psittaci*.[40] Serological testing was performed for the presence of antibodies to *Mycoplasma pneumoniae*, *Coxiella burnetii*, *Chlamydophila pneumoniae* / *psittaci* or respiratory viruses (adenovirus, influenzavirus A and B, parainfluenzavirus 1, 2 and 3 and the respiratory syncytial virus) (Serodia, Bipharma, Fujirebio Inc, Tokyo, Japan). A fourfold increase in antibody titre was considered as significant. Pharyngeal samples were taken for viral culture.

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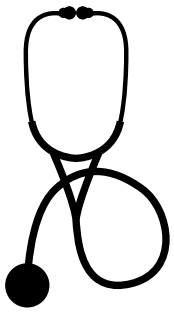

# Chapter 12

# Biomarkers define the clinical response to dexamethasone in community-acquired pneumonia

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## Abstract

### Background

Adjuvant dexamethasone treatment in patients with community-acquired pneumonia (CAP) can reduce length of hospital stay. Whether there are subgroups of patients that especially might benefit from corticosteroids is unknown. We hypothesised that a discrepancy between systemic inflammation and cortisol level can define a subgroup that lacks a sufficient cortisol response during CAP, and therefore particularly might benefit from corticosteroids.

### Methods

A secondary analysis was performed on data from hospitalised patients with CAP, randomised to an adjunctive four-day course of dexamethasone (5 mg daily) or placebo. Subgroups were made based on plasma cytokine levels (interleukin-6 (IL-6), IL-8, monocyte chemotactic protein-1 (MCP-1)) and cortisol on presentation. Intensive care unit (ICU) admission and mortality were assessed.

### Results

275 Patients (131 dexamethasone, 144 placebo) were analysed. In the subgroup of patients (n=46) with a high cytokine response (defined as IL-6≥92.5 pg/mL, IL-8≥14.8 pg/mL and MCP-1≥1154.5 pg/mL) and a discrepantly low cortisol (lowest 50%), dexamethasone treatment was associated with a significant decrease on a combined endpoint of mortality/ICU admission, as compared with placebo (0% vs. 43%, $p<0.01$).

### Conclusion

In patients presenting with a high pro-inflammatory cytokine response but a discrepantly low cortisol, adjuvant dexamethasone treatment was associated with a significant decrease in mortality and ICU admission.

## Introduction

Community-acquired pneumonia (CAP) is a common disease that is still characterised by significant morbidity and mortality, despite adequate antibiotic treatments.[1] Last years, research has focused on adjuvant treatment strategies. A promising adjuvant therapy is the administration of corticosteroids during CAP. Corticosteroids are thought to exert their beneficial effect during inflammation by modulation of the immune response, or by relief of adrenal insufficiency.[2] Previously, we showed that dexamethasone can reduce length of hospital stay when added to antibiotic treatment in non-immunocompromised patients with CAP.[3]

Cortisol is an important regulator of inflammation. In general, the cortisol production increases during infection. Stress induced by severe illness strongly activates the hypothalamic-pituitary-adrenal (HPA) axis, which is an integral part of the host response to infection.[4] The increased cortisol level has several important effects on metabolism and the cardiovascular system, and exhibits anti-inflammatory and immunosuppressive effects. The latter are characterised by the dampening of pro-inflammatory cytokines, chemokines and other inflammatory mediators. Furthermore, cortisol enhances the release of anti-inflammatory factors.[5]

Dysfunction of the HPA axis in critically ill patients, frequently referred to as critical illness-related corticosteroid insufficiency (CIRCI), has recently been defined as a random total cortisol of <10 µg/dL or a delta serum cortisol of <9 µg/dL after administration of 250 µg synthetic adrenocorticotrophic hormone (ACTH, corticotropin test).[6] Critically ill patients lose their circadian rhythm in cortisol levels, and therefore cortisol measurements in these patients can be performed at a random time of the day.[7]

Because cortisol is needed to control inflammation, an insufficient cortisol response during infection is expected to have an unfavourable impact on clinical outcome. This has been confirmed in patients with septic shock, in which CIRCI was associated with a higher risk of death.[8] Although not all studies have shown a beneficial effect of corticosteroids in these patients, therapy with low-dose corticosteroids is common in septic shock.[9,10] Surprisingly, low cortisol levels (<10 µg/dL) were not associated with worse outcomes in patients with CAP.[11] Furthermore, to date, no favourable effect was found of corticosteroids on mortality and ICU admission in the overall CAP population.[12]

Whether there are specific subgroups of patients with CAP who might benefit from corticosteroid treatment is unknown. The correlation between systemic inflammation, as measured by cytokine response and cortisol level has not been investigated thus far. We hypothesised that a discrepancy between systemic inflammation and cortisol level may identify a subgroup of patients who lack a sufficient cortisol response in CAP, and therefore might benefit from dexamethasone administration.

## Methods

### Patients

We performed a *post-hoc* analysis on data from patients with CAP enrolled in a study on the effect of dexamethasone on length of hospital stay. The details of the study population and design have been described previously.[3] In short, from November 2007 until September 2010, adult patients with confirmed pneumonia were prospectively enrolled in the study at the St. Antonius Hospital in Nieuwegein or at the Gelderse Vallei Hospital in Ede, both teaching hospitals in the Netherlands. Patients who were immunocompromised, on immunosuppressive therapy (including oral corticosteroids), or who required immediate admission to the intensive care unit (ICU) were excluded. For the present analysis, patients using oral contraceptives or ketoconazole were excluded as well. The need for ICU admission and in-hospital mortality were assessed.

### Study design

Patients were randomised to receive a bolus of 5 mg (1 mL) of dexamethasone intravenously (IV) or 1 mL of sterile water IV on the emergency unit. On the following three days the patients received either IV dexamethasone 5 mg (1 mL) or sterile water 1 mL once a day. The local Ethics Committee approved the study and informed consent was obtained from all patients.

### Laboratory tests

The total plasma cortisol level was measured with a solid-phase competitive ELISA (Calbiotech, Spring Valley, USA) on the day of presentation, before administration of the first dose of dexamethasone. Systemic concentrations of interleukin-6 (IL-6), IL-8 and monocyte chemotactic protein-1 (MCP-1) were measured on admission using Milliplex multi-analyte profiling (Millipore, Billerica, USA). Furthermore, serum albumin and concentrations of C-reactive protein (CRP) were measured on presentation (Roche Diagnostics, Mannheim, Germany).

### Statistical analyses

All statistical analyses were performed using SPSS 15.0 (Chicago, USA). A two-sided p-value of $<0.05$ was considered to be statistically significant. Differences in categorical variables were analysed with the Chi-square test or Fisher's exact test, and differences in continuous data were analysed with Student's t-test or Mann-Whitney U-test, as appropriate.

Correlation analyses were performed by Spearman's rank correlation. To evaluate which patients benefit most from dexamethasone therapy, all patients

were classified based on the severity of systemic inflammation and total cortisol level. Severe systemic inflammation was defined as a high cytokine response on admission. We selected IL-6, IL-8 and MCP-1 for the analysis, because these are the most important pro-inflammatory cytokines during infection.[13-15] Optimal cut-off points for IL-6, IL-8 and MCP-1 to predict the combined endpoint of mortality/ICU admission were determined using receiver operator characteristics (ROC) curve analysis and the Youden's index.[16] At first, all patients were divided into two subgroups: patients with all three cytokines above the cut-off values (high cytokine response) versus all other patients (low cytokine response). Subsequently, each group was further subdivided into 'low' and 'high' cortisol level, based on the median cortisol level of each group. Finally, for all four subgroups, the effect of dexamethasone on a combined endpoint of mortality and ICU admission was evaluated.

## Results

### Study population

A total of 304 patients were enrolled in this study. After the exclusion of 15 women who were using oral contraceptives and 14 patients missing a day 0 cortisol value, 275 patients were analysed. 131/275 patients (48%) were randomised to dexamethasone and 144/275 (52%) to placebo. The baseline patient characteristics are described in *table 1*. There were no significant differences in median cortisol levels between patients with inhaled corticosteroids at home (n=25, cortisol 23.2 µg/dL, IQR 15.5 – 48.5) and without (n=250, cortisol 22.6 µg/dL, IQR 14.9 – 39.8) (p=0.44).

During their hospital stay, 16/275 patients (5.8%) died and 17/275 patients (6.2%) were admitted to the ICU. On admission, 27/275 patients (9.8%) had a cortisol <10 µg/dL. Mean serum albumin did not differ between patients with cortisol <10 µg/dL and >10 µg/dL (p=0.34). In patients with cortisol <10 µg/dL, 19/27 patients (70%) had a pneumonia that ranked as pneumonia severity index (PSI) class I–III, compared to 120/248 patients (48%) with cortisol >10 µg/dL (p=0.03). None of the patients with cortisol <10 µg/dL died, compared to 16/248 patients (6.5%) with cortisol >10 µg/dL (p=0.17). In the patients with cortisol <10 µg/dL, 2/27 patients (7.4%) were admitted to the ICU, compared to 15/248 patients (6.0%) with cortisol >10 µg/dL (p=0.78).

**Table 1.** Baseline characteristics of 275 patients with CAP

| Characteristics | Placebo group (n = 144) | Dexamethasone group (n = 131) |
|---|---|---|
| **Sex, male** | 84 (58.3) | 79 (60.3) |
| **Age, years** | 63.9 (17.8) | 66.5 (17.1) |
| **Comorbidities** | | |
| Neoplastic disease | 10 (6.9) | 9 (6.9) |
| Liver disease | 0 (0) | 2 (1.5) |
| Congestive heart failure | 23 (16.0) | 24 (18.3) |
| Renal disease | 10 (6.9) | 19 (14.5) |
| Diabetes mellitus | 21 (14.6) | 20 (15.3) |
| COPD | 13 (9.0) | 17 (13.0) |
| **Pneumonia Severity Index Score** | 90.0 (35.8) | 97.6 (35.1) |
| **Pneumonia Severity Index risk class** | | |
| Class I | 18 (12.5) | 11 (8.4) |
| Class II | 31 (21.5) | 23 (17.6) |
| Class III | 31 (21.5) | 23 (17.6) |
| Class IV | 43 (29.9) | 50 (38.2) |
| Class V | 21 (14.6) | 24 (18.3) |
| **Laboratory findings** | | |
| C-reactive protein, mg/L | 212.1 (137.5) | 223.3 (146.2) |
| Total cortisol, µg/dL | 223.1 (143.6-395.0) | 236.4 (151.8-410.9) |
| Albumin, g/L | 41.8 (7.4) | 42.2 (8.3) |
| **Outcome** | | |
| Hospital mortality | 8 (5.6) | 8 (6.1) |
| ICU admittance | 10 (6.9) | 7 (5.3) |

Data are presented as number (%), mean (SD) or median (IQR)

## Correlation between cytokine response and cortisol level on admission

In 265 (96%), 272 (99%), and 274 (100%) of the patients, IL-6, IL-8 and MCP-1, respectively, were measured upon admission. As expected, significant correlations were found between cortisol and IL-6 ($r = +0.486$; $p<0.01$), IL-8 ($r = +0.440$; $p<0.01$), and MCP-1 ($r = +0.376$; $p<0.01$), indicating that cortisol changes in parallel with these cytokines. In patients with cortisol <10 µg/dL, serum IL-6, IL-8, MCP-1 and CRP were significantly lower compared to patients with cortisol >10 µg/dL, as shown in *figure 1*.

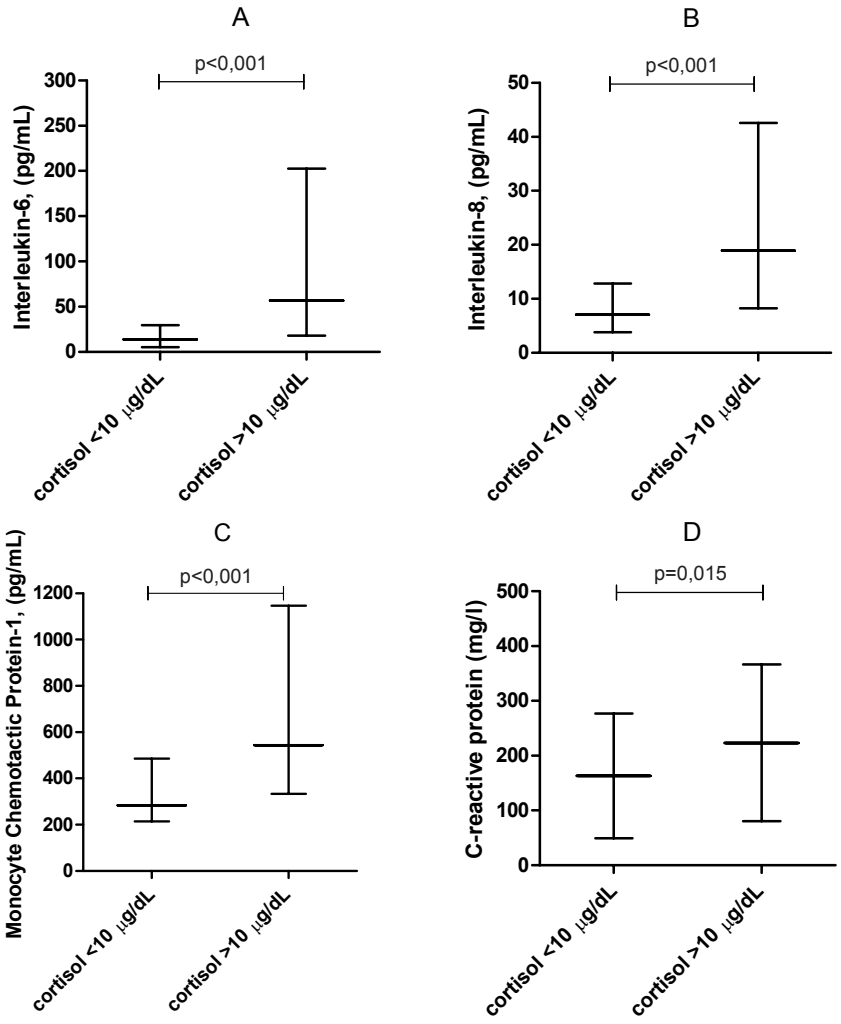


**Figure 1.** Comparison of the median levels and interquartile ranges of Interleukin-6 (panel A), Interleukin-8 (panel B), monocyte chemotactic protein-1 (panel C) and the mean levels and standard deviations of C-reactive protein (panel D) between patients with cortisol <10 µg/dL and >10 µg/dL.

## Cytokine response, cortisol level and the effect of dexamethasone

In total, 46/275 patients (17%) had a high cytokine response (defined as IL-6 ≥92.5 pg/mL, IL-8 ≥14.8 pg/mL and MCP-1 ≥1154.5 pg/mL), indicating severe systemic inflammation. From these patients, 11/46 patients (24%) died or were admitted to the ICU, compared to 17/225 patients (7.6%) in the low cytokine response group (p<0.01).

In patients with a high cytokine response, the median cortisol was 47.9 µg/dL (IQR 20.1 – 72.2). We evaluated whether patients with a high cytokine response and discrepantly low cortisol (<47.9 µg/dL) benefited more from dexamethasone therapy than patients with a high cytokine response and high cortisol (>47.9 µg/dL). As shown in *figure 2*, in patients with a high cytokine response and discrepantly low cortisol, treatment with dexamethasone was associated with a significant decrease on a combined endpoint of mortality and ICU admission, compared with placebo (0% vs. 43%, p<0.01). In patients with a high cytokine response and high cortisol, this favourable effect of dexamethasone on mortality/ICU admission was not found, as compared with placebo (30% vs. 39%, p=0.67). Serum albumin did not differ between these two subgroups (41.4±8.2 g/L vs. 40.3±10.9 g/L) (p=0.70).
When we used the 25$^{th}$ percentile as a cut-off value for low cortisol (<20.1 µg/dL), the decrease on the combined endpoint of mortality/ICU admission caused by dexamethasone as compared to placebo was even higher, but the group was too small (n=12) to reach a significant effect (0% vs. 50%, p=0.091).

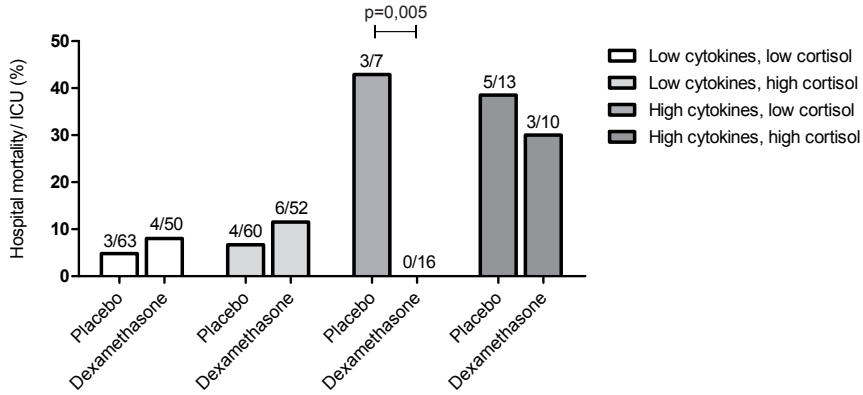


**Figure 2.** All patients were classified into four groups, based on the combination of cytokine response (high or low) and cortisol level (high or low) on admission. High cytokine response was defined as a combination of IL-6 ≥92.5 pg/mL, IL-8 ≥14.8 pg/mL and MCP-1 ≥1154.5 pg/mL. The patients not meeting this combination were defined as having a low cytokine response. For both groups, the median cortisol level was determined. Based on the median, patients were classified into subgroups with high (above the median) or low (below the median) cortisol on admission. The median cortisol level was 47.9 µg/dL in the high cytokine response group and 20.6 µg/dL in the low cytokine response group. The effect of dexamethasone treatment on a combined endpoint of mortality/ICU admission is shown for all four combinations.

In patients with a low cytokine response (n=229), the median cortisol was 20.6 µg/dL (IQR 14.3 – 32.8). In contrast to patients with a high cytokine response, dexamethasone had no beneficial effect on mortality/ICU admission in patients with a low cytokine response and low cortisol (<20.6 µg/dL).

Because a single parameter as indicator for the inflammatory response would be easier to use in clinical practice, we tried to replace the combination of three cytokines by CRP, a single cytokine or high PSI score (PSI class IV and V) on presentation. However, in those subgroups no significant clinical effect was found from dexamethasone treatment (data not shown).

## Discussion

In this study, we confirm that a low plasma cortisol <10 µg/dL on admission is not associated with an adverse outcome in patients with CAP. In a *post-hoc* subgroup analysis of patients with a high cytokine response and a discrepantly low cortisol level on admission, we showed, however, a beneficial effect of dexamethasone on a combined endpoint of hospital mortality and ICU admission.

Because cortisol is an important regulator of inflammation, an insufficient cortisol response during infection is expected to result in excessive, ongoing inflammation, which may be associated with a poor outcome. This is confirmed in patients with septic shock, in which CIRCI was associated with an adverse outcome.[8] However, in our study of patients with CAP, cortisol <10 µg/dL was not associated with adverse outcome. This is consistent with a recent meta-analysis of the existing literature regarding patients with CAP.[11] A possible explanation for the difference between septic shock and CAP might be that CAP patients with cortisol <10 µg/dL have a lower disease severity rather than corticosteroid insufficiency. Indeed, in our series, most of the patients with cortisol <10 µg/dL had a lower cytokine response and were more frequently classified in PSI classes I–III. In these cases, the low cortisol levels seem appropriate because of non-severe CAP.

In a subgroup analysis, in which cytokine profiles were correlated to cortisol levels, we showed that patients with a high cytokine response but a discrepantly low cortisol level benefited most from dexamethasone on a combined endpoint of mortality and ICU admission. This suggests that these patients lack a sufficient adrenal response and are probably in need of extra glucocorticoids to balance the intense pro-inflammatory response. Based on the present findings, we believe that a total cortisol level on admission should be interpreted in conjunction with the pro-inflammatory cytokine profile to identify the patients with an insufficient cortisol response during CAP.

The mechanisms leading to dysfunction of the HPA axis during severe illness are complex and incompletely understood. Decreased production of the hormones corticotropin-releasing hormone (CRH), ACTH, and cortisol, as well as tissue glucocorticoid resistance, may play a role in the development of adrenal insufficiency.[17] Corticosteroids potently inhibit inflammation by down-regulating pro-inflammatory cytokine gene transcription.[18,19] Besides this anti-inflammatory effect, the administration of synthetic corticosteroids can replenish the low tissue levels of endogenous cortisol during dysfunction of the HPA axis. Moreover, synthetic corticosteroids can (partially) overcome peripheral tissue glucocorticoid resistance.[20]

Our study has several strengths compared to previous studies. This is the first study that measures and correlates cytokine profiles and cortisol levels in patients with CAP. Due to the randomised controlled design of the study, we were able to assess the effect of a standardised dose of dexamethasone on outcome, compared to placebo.

Our study also had limitations. First, this is a *post-hoc* analysis of data generated in a recently published randomised controlled trial.[3] Therefore, the results of this study should be considered hypothesis-generating; a new prospective clinical trial must be conducted to confirm our findings. Second, due to the design of the study, only random total cortisol levels could be assessed. In the recent literature, CIRCI was defined as cortisol <10 µg/dL or delta serum cortisol <9 µg/dL after the administration of 250 µg synthetic ACTH (corticotrophin test).[6] As corticotropin tests were not performed in our study, we were unable to identify patients with CIRCI, defined as delta serum cortisol <9 µg/dL after administration of 250 µg synthetic ACTH. This did not influence our conclusion with respect to the absence of an adverse outcome in patients with cortisol <10 µg/dL. However, we might have missed an additional subgroup of patients with corticosteroid insufficiency who might also benefit from dexamethasone. Third, it is unclear whether the circadian rhythm of cortisol was lost in all patients. As a consequence, we might have found lower cortisol levels in some patients. Finally, in our analysis to better define the patients who benefited most from dexamethasone treatment, we used the median cortisol level to discriminate between low and high cortisol. The number of patients was too small to allow proper analyses with other cortisol cut-off values.

In conclusion, this *post-hoc* analysis confirmed that a total plasma cortisol <10 µg/dL in patients with CAP is not associated with adverse outcomes. Most of these patients did have non-severe disease instead of corticosteroid insufficiency. We further observed that in a subgroup of patients with severe systemic inflammation, as indicated by a high cytokine response, and a discrepantly low cortisol on admission, dexamethasone treatment was

associated with a significant decrease on a combined endpoint of mortality and ICU admission. This correlation between cytokine response and cortisol level might better reflect corticosteroid insufficiency during CAP and can help to identify patients who will benefit most from dexamethasone treatment. Further prospective clinical studies are needed to confirm this hypothesis.

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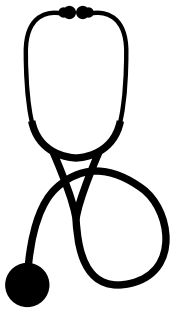

# Chapter 13

## Summary, general discussion and perspectives

## Summary

Despite advances during the last few decades in the prevention, diagnosis and treatment of community-acquired pneumonia (CAP), its incidence remains high. Many patients are hospitalised, and some die from pneumonia.
Aside from the burden of disease for the individual patient, healthcare costs due to CAP are considerable and will only rise due to the ageing of Western populations. In this thesis, strategies are described for improvement of the quality of clinical management in patients with pneumonia and for the reduction of the healthcare burden. The focus is on the prevention of pneumonia, a better understanding of the immune response in CAP, further development of biomarkers, and adjunctive therapies.

**Susceptibility to and prevention of CAP**
Recent studies have suggested that gastric acid-suppressive medication may increase the risk of CAP. In **Chapter 2**, a population-based case-control study is presented on the relationship between proton pump inhibitor (PPI) treatment and CAP. Of patients admitted to the hospital for pneumonia, 435 were matched to control patients from the PHARMO database in a 1:4 ratio. Overall, after adjustment for confounding factors, the risk of CAP was higher in patients receiving PPI treatment (odds ratio (OR): 1.6; 95% CI 1.2 – 2.2). This risk was highest shortly (≤15 days) after the initiation of PPI treatment (OR: 3.1; 95% CI 1.1 – 8.8). Because of this seemingly contradictory timing effect, CAP patients who recently started PPI treatment were examined. It became clear that protopathic bias (prescribing of PPI treatment for early symptoms of pneumonia) did not explain the observed increased risk. Additionally, the study of the causative microorganism of CAP did not show an increase in the frequency of either oropharyngeal or gastrointestinal bacteria in patients using PPIs.

**Immune response in CAP**
Numerous genetic variations (polymorphisms and haplotypes) are found in genes of the innate immune system. Some of these variations were found to be associated with CAP susceptibility or outcome. For example, the interleukin (IL)-6–174 C/C polymorphism was associated with an increased susceptibility to CAP due to a decreased inflammatory response. In the complement system as well, single nucleotide polymorphisms (SNPs) have been found, in particular within the MBL and ficolin genes. The +6359C>T SNP leading to the Thr236Met amino acid alteration in the ficolin-2 gene was associated with decreased substrate binding. These results suggest that there is a genetic basis for susceptibility to and outcome of CAP.

In **Chapter 3**, the cytokine response in a cohort of 201 non-immunocompromised patients with CAP is described. IL-6 and IL-8, together with IL-1Ra and IL-10, act as acute-phase proteins in CAP. IL-6 is a more accurate biomarker for the follow-up of CAP than C-reactive protein (CRP).
The systemic cytokine response is partly determined by the causative microorganism. IL-6 and IL-1Ra responses are significantly higher in patients with pneumococcal pneumonia than in patients with non-pneumococcal pneumonia or pneumonia caused by an atypical bacterium. Our experimental evidence has shown that SNPs in the IL-6, IL-8, and IL-10 genes (including haplotypes) do not influence cytokine production, and no association was found between cytokine polymorphisms and clinical outcomes. In a non-randomised setting, the cytokine response was shown to be reduced by the use of corticosteroids in patients with CAP.

In addition to the inflammatory response as discussed in Chapter 3, the complement system is an important component of the innate immune system. It is known that mannose-binding lectin (MBL) and ficolin-2 are important in the activation of the complement system and that both are well capable of binding to *S. aureus*. In **Chapter 4**, the role of polymorphisms in the genes of these two lectins in patients with *S. aureus* peritonitis on continuous ambulatory peritoneal dialysis (CAPD) is studied. In our cohort of 105 patients on CAPD, the +6359C>T SNP in the ficolin-2 gene was associated with an increased risk of developing staphylococcal peritonitis. Patients with the +6359T/T genotype had more frequent peritonitis than patients with the +6359C/C genotype (OR: 5.57; 95% CI 1.29 – 24.05). SNPs in the MBL gene are not associated with an increased risk of staphylococcal peritonitis.

In the earlier literature, ficolin-2 insufficiency appeared to be associated with infectious respiratory disease in children. However, in a limited number of in vitro studies performed thus far, little to no binding of MBL and ficolin-2 was found on the surface of pneumococci. The role of MBL and ficolin-2 in the opsonisation of pneumococci is therefore considered limited. This led to the study described in **Chapter 5** in which binding to the pneumococci of MBL, ficolin-2 and subsequently MASP-2 was explored. MASP-2 binding to pneumococci was found in 19/32 patients (59%), MBL binding in 13/32 patients (41%) and ficolin-2 binding in 28/32 patients (88%). In a subgroup of patients in which binding of MBL, but not ficolin-2, on the pneumococci was observed, no significant MASP-2 binding was seen. MASP-2 binding was independent of *S. pneumoniae* serotype. This study showed that pneumococci can bind MASP-2 and that ficolin-2 seems to be the overriding MASP-2 activator.

Improvement of CAP diagnosis and therapy is not based only on a better understanding of the innate immune response but also on an analysis of the response of the adaptive immune system, both of which can result in new insights. In **Chapter 6**, a quantitative multiplex immunoassay for the measurement of serotype-specific antibody concentrations at different time points after the onset of CAP is described. This assay was used to identify 14 serotype-specific anti-capsular polysaccharide IgG antibodies. In 28% of patients in which no causative agent for CAP could be identified, a serotype-specific anti-pneumococcal antibody response was found. The estimated contribution of the pneumococcus in patients with an unidentified causative agent of CAP was calculated to be 57% (95% CI 36 – 86%). This method is not useful at the moment of diagnosis because the immune response takes 5-10 days to develop fully. However, considering the estimated contribution the pneumococcus, the use of single beta-lactam therapy may be indicated in most patients with CAP. Moreover, this estimated contribution may contribute to the design of vaccination schedules for patients at risk with the 23-valent polysaccharide vaccine.

**Biomarkers**

A biomarker is a substance that can be used as an indicator of a biological state. In the case of CAP, the individual components of the immune response to an infecting agent have the potential to serve as biomarkers for disease severity and progression. In this context, the above described cytokines IL-6, IL-8, and MCP-1, and the complement components MBL and ficolin-2, can be considered as immune biomarkers for CAP. The usefulness of two possible biomarkers outside of the immune system in patients with CAP to predict severity and risk for adverse outcome are explored. First, in **Chapter 7**, the usefulness of serum angiotensin I-converting enzyme (ACE) activity at the time of hospital admission as a prognostic marker is assessed. Serum ACE originates mainly from lung micro-vessels, and for that reason, it has been studied as a potential biomarker for various pulmonary disorders. In a previous study on CAP, a negative correlation between serum ACE activity and a combination of physiological parameters was observed[1] as well as a trend towards worse outcome in patients with low serum ACE activity at the time of hospital admission. That study was not able to test the association of ACE activity with outcome of CAP satisfactorily, potentially due to the relatively small sample size. Therefore, in the study presented in Chapter 7, a second cohort was combined with the first cohort. In that study, the substantial decrease in serum ACE activity during episodes of pneumonia was confirmed, but a significant prognostic value for ACE activity regarding clinical outcome was not found. Interestingly, low serum ACE activity

<24 U/L at the time of hospital admission appeared to be a strong indicator of the presence of bacteremia (adjusted OR: 3.93; 95% CI 1.57 – 9.87). Combined with CRP levels and leukocyte counts, serum ACE activity can become useful for the identification of bacteremia before the outcome of blood cultures is known.

Second, in **Chapter 8**, the incidence and predictive value of proteinuria as a biomarker for outcome in patients with CAP is presented. Recent literature has shown that in patients with chronic kidney disease, the presence of proteinuria predicts outcome independent of renal function.[2] Strikingly, even in the general population, proteinuria predicts morbidity and mortality.[3] However, the predictive value of proteinuria during an episode of acute illness has not yet been reported. In 319/496 (64%) patients studied, a urine sample was taken on the day of admission, and proteinuria was frequently present in these patients (62%). Proteinuria on admission was, in contrast to other known criteria for acute kidney failure, independently associated with increased length of hospital stay in CAP patients (adjusted OR: 1.38; 95% CI 1.08 –1.75).

**Adjunctive therapy**

The primary goal in the treatment of CAP is antibiotic therapy as soon as possible. However, antibiotic resistance against the most common bacteria is increasing. Furthermore, it is known that the severity of pneumonia is related to the extent of the inflammatory response. Therefore, it is important (and in the future may be necessary) to search for adjunctive non-antibiotic therapies for patients with CAP.

In **Chapter 9**, the current literature concerning the various options for supportive therapy of patients treated with otherwise effective antibiotics is reviewed. In view of increasing resistance, these supportive therapies may become the only option. However, corticosteroids, activated protein C, and immunoglobulins most likely could not be used as monotherapy. As adjunctive therapy, complement including MBL and Toll-like receptor agonists and antagonists are attractive options but warrant additional studies because sufficient evidence is not currently available. Corticosteroids are widely available and can be used as adjunctive therapy in patients with CAP complicated by severe sepsis or septic shock. In contrast to the large number of studies on sepsis and septic shock, there are only a few controlled trials that investigated corticosteroids as adjunctive treatment to antibiotics in patients with CAP, and these trials have produced inconsistent results. Therefore, in **Chapter 10**, a randomised placebo-controlled trial (RCT) using corticosteroids as an adjunctive treatment to antibiotics in patients with CAP is described. Hospitalised, non-immunocompromised

patients with CAP were randomly assigned to an intervention group of an adjunctive four-day course of dexamethasone (5 mg once a day) or to a placebo group. Primary outcome was length of hospital stay. A total of 304 patients (153 in the placebo group and 151 in the intervention group) were analysed. The median length of stay was 6.5 days for patients in the dexamethasone group and 7.5 days in the placebo group (p=0.048). Overall, CRP and IL-6 levels declined faster in patients on dexamethasone than in patients in the placebo group. The quality of life with regard to social functioning was better on day 30 in the dexamethasone group than in the placebo group. Hyperglycaemia was more often recorded in the dexamethasone group, but severe adverse events were rare and did not differ between the groups.

To further elucidate which patients benefit most from corticosteroids, the cytokine response of 304 patients in an RCT was evaluated in **Chapter 11**. The overall decrease in cytokine levels was more rapid in patients with CAP treated with dexamethasone in addition to antibiotic treatment than in patients without dexamethasone. Interestingly, after construction of a model with IL-6, IL-8 and MCP and the selection of all patients in which all three cytokines (IL-6 ≥ 4.53 mg/dL, IL-8 ≥3.79 mg/dL) and MCP ≥6.70 mg/dL) were elevated, patients with a high cytokine response benefited most from dexamethasone in terms of hospital mortality and ICU admission. In these patients, 2 of the 24 patients in the dexamethasone group died or were transferred to the ICU (8.3%) compared with 8 of the 17 patients (47%) in the placebo group (p<0.01, OR: 0.10; 95% CI 0.02 – 0.58).

In critically ill patients, inadequate adrenal response (cortisol <10 µg/dL or a change in serum cortisol of <9 µg/dL after the administration of 250 µg synthetic ACTH) is associated with increased risk of death. This syndrome is referred to as critical illness-related corticosteroid insufficiency (CIRCI). It is suggested that these patients benefit most from corticosteroid treatment. In **Chapter 12**, it is shown that in patients with CAP, a cortisol level <10 µg/dL was not found to be associated with poor outcomes. Furthermore, patients with high cytokine responses and unexpectedly low cortisol levels (<50% of the patients with high cytokine responses) were shown to benefit more from dexamethasone therapy. Thirty-one percent of the patients in the placebo group died compared with 0% in the dexamethasone-treated group. Therefore, a cortisol level <10 µg/dL used in the current part of the definition of CIRCI is inaccurate for patients with CAP. Most patients with cortisol levels <10 µg/dL have mild disease rather than adrenal insufficiency. Furthermore, in patients with high cytokine responses and unexpectedly low cortisol levels on admission, dexamethasone treatment

reduced mortality and ICU admission. This correlation between cortisol levels and cytokine response might better reflect patients who benefit from dexamethasone treatment.

## General discussion and perspectives

The studies presented in this thesis provide insight into possible interventions to improve the clinical management of CAP. Yet, many questions remain unanswered. In this general discussion, the implications of the studies presented in this thesis and the opportunities for future research are discussed.

### Susceptibility to and prevention of CAP

#### *Is it possible to prevent the development of CAP?*

In chapter 2, it is shown that PPI use increases the risk of CAP, especially in the first 15 days after the initiation of treatment. The mechanism by which PPI treatment causes pneumonia, however, is unknown. Neither protopathic bias (when a treatment for the first symptoms of a disease appears to cause the disease) nor shifts in microbial aetiology seem to explain this association. It should be mentioned that only the microbiological aetiology of CAP between patients who were or were not using PPI were compared. We did not study the microbial composition of the upper respiratory tract.

Further future research also could be directed towards other PPI properties such as the interaction with the immune system. It is known that PPIs impair natural killer cell activity, but only *in vitro* at concentrations not reached during oral or intravenous treatments.[4] Furthermore, PPIs inhibit the expression of adhesion molecules and thereby attenuate endothelial leukocyte interactions.[5] Additionally, PPIs impair the production of reactive oxygen intermediates by neutrophils, which can lead to decreased bactericidal activity.[6] While all these data may suggest a negative effect of PPIs on the ability of the host to adequately combat infection, they do not provide an explanation for the observation that the greatest risk for CAP is during the first period of PPI use.

Disregarding the underlying mechanism, our finding of an increased risk shortly after initiation of treatment raises several concerns about the use of PPIs. The current guidelines for clinicians on the prescription of PPI treatment are accurate, and there is no doubt that patients who are at risk for gastrointestinal complications should use PPI treatment.[7] However, since September 2010, omeprazole and other PPIs are available over-the-counter in the Netherlands.[8] Advertisement for these drugs on television and in written media will probably increase their overall use in patients who are not at risk for gastrointestinal complications. Moreover, it is likely that patients will use this medication for only a short period of time until the gastric disturbances subside and restart use whenever complaints return. Although we have not specifically studied repeated intermittent episodes of PPI use, this type of usage may result in an increased risk of CAP because the risk is highest in the 15 days after the

start of PPI treatment. Although there are also beneficial effects of over-the-counter available PPIs (less gastric complaints during over-the-counter NSAID use), patients and clinicians should be informed about the risks of using such medications.
Additionally, over-the-counter availability of PPIs complicates future studies. Retrieving all dispensed prescriptions from the pharmacy will not be sufficient to identify all PPI use. This could lead to undifferential misclassification and a finding of a null effect.

***Should vaccination against S. pneumoniae be extended?***
In most epidemiological studies, the incidence of *S. pneumoniae* as the causative agent of CAP was 25 – 35%, while in 40 – 50% of patients, no causative agent was found.[9-12] In chapter 6, a new diagnostic method was described; this method showed that the estimated contribution of *S. pneumoniae* in patients with an unidentified causative agent was approximately 57% (95% CI 36 – 86%). This finding stresses the importance of vaccination against pneumococci in the prevention of CAP. However, the indication for pneumococcal vaccination varies widely and remains a topic of debate.[13-16]
In an on-going study (CAPITA), the efficacy of a 13-valent pneumococcal conjugate vaccine for CAP is being investigated in the Netherlands.[17] Should the use of this vaccine in the elderly be effective in reducing the incidence of vaccine-serotype pneumococcal CAP, it may lead to implementation of this vaccine from the age of 65 years onward. This could lead to a shift in the pneumococcal serotype distribution in adults and the elderly as has occurred in infants and young children.[18] It is difficult to predict whether this would lead to replacement disease, and if so, to replacement with other pneumococcal serotypes or other bacterial species. In the latter case, this would also have an impact on microbiological diagnosis and antibiotic use.

**Immune response in community-acquired pneumonia**

***What is the role of complement in the defence against pneumococci?***
The innate immune system comprises germline-encoded mechanisms that defend the host against infection. The innate immune system depends on receptors that recognise repeated patterns of molecular structures on the surface of microorganisms. This results in cytokine recruitment and complement activation which leads to a non-specific attack of the microbial cell membrane and eventually, in most cases, resolution of the infection.[19-21] The adaptive immune system is able to recognise the enormous variety of microbial antigens and is believed to be more specific and sophisticated. The innate immune system constitutes an evolutionarily older defence strategy and is the dominant

immune system found in insects and in primitive multicellular organisms. All animal forms since the ancestors of cartilaginous fish have retained their innate immune systems and developed well-working adaptive immune systems.[22] An example of the importance of the innate immune system in host defence against pneumococci is found in *Drosophila*. The fruit fly lacks an adaptive immune system but is highly resistant to microorganisms.[23] This suggests that the innate immune system in itself is capable of mediating the necessary defence mechanisms against infections with microorganisms including pneumococci.[24] The innate immune system acts fast and can strengthen the adaptive immune response.

Genetic variability affecting the host innate immune response might influence the susceptibility and outcome of infection. In our cohort of 105 patients on CAPD, the +6359C>T SNP leading to the Thr236Met amino acid alteration in the ficolin-2 gene was associated with an increased risk of developing staphylococcal peritonitis.
Pneumococcal pneumonia severity varies widely from almost asymptomatic to septic shock and organ failure. In our study on pneumococcal pneumonia, the patient numbers were too small to detect the influences of genotype on susceptibility or disease severity. However, in most patients the lectin pathway of complement was activated during pneumococcal pneumonia, with ficolin-2 binding to pneumococci as the overriding factor and a less important role for MBL binding. In other studies, homozygous-deficient MBL patients were at more risk for developing invasive pneumococcal disease compared to MBL sufficient patients.[25-27] This suggests that low levels of complement might result in reduced opsonisation, which favours the survival of *S. pneumoniae* early in the course of invasion.[25] The role of ficolin-2 insufficiency in the susceptibility to or severity of pneumococcal pneumonia may be of even greater importance compared with MBL deficiency but has not yet been tested. Further research should demonstrate the role of ficolin-2 in pneumococcal disease.

Although the innate immune system plays a role in pneumonia, it is questionable whether a deficiency in this system solely would have clinical implications. Replacement of MBL has been used as a therapy in Scandinavian patients with MBL deficiency and repeated infections and might be beneficial.[28] However, pneumococcal pneumonia occurs in most patients as a single entity, and it is difficult to predict who is most at risk. Because it is feasible that the innate immune system is most important early in the infection, replacement therapy during admission of a patient with CAP to the hospital would probably be too late. In patients on CAPD with a continuous risk of infection, replacement

therapy might be more promising. In contrast with MBL, replacement of ficolin-2 or medication to increase ficolin-2 levels has not been tested at all. In addition to the possible beneficial effects of replacement of MBL and ficolin-2, care should be exercised to guard against the introduction of auto-immune disease due to complement activation; for example, the atypical forms of haemolytic-uremic syndrome (HUS).[29-31]

Until further research in exploring the role of MBL and ficolin-2 replacement therapy is conducted, vaccination is a potentially more cost-effective method to protect patients with MBL and ficolin-2 deficiencies of the innate immune system from pneumococcal pneumonia.

**Biomarkers**

***Is it time to revisit the pneumonia severity index (PSI)?***

As argued above, biomarkers can be helpful in the management of CAP to assess disease severity and allow for timely initiation of adequate treatment. In emergency departments, the PSI score is often used for this purpose. Although the PSI score, developed in 1997 by Fine *et al*., is highly appreciated by clinicians and is widely used, it has some disadvantages[32]. First, it is time consuming to calculate, and the scoring system is too complicated to memorise. Second, the PSI score was developed to predict 30-day mortality in patients with CAP, not to assess severity or predict morbidity of CAP at the moment of admission. Because the PSI score is highly influenced by age, young patients even with severe illness will consequently have low scores. Therefore, it is important to search for new biomarkers that could improve (or replace) the PSI score to make it more useful in assessing CAP severity. When a clinician is able to better determine morbidity or severity of illness at the time of admission, measures can be taken to improve the quality of clinical management.

In this thesis, the role of serum ACE activity and proteinuria as possible new biomarkers for CAP are explored. Serum ACE activity in patients with CAP has no prognostic value regarding outcome measures. However, CAP patients with serum ACE activity <24 U/L at the time of hospital admission have an almost four-fold increased risk for the presence of bacteremia compared with patients with normal serum ACE activity. In combination with CRP and leukocyte counts, low serum ACE provides a *post*-test odds of 1 for bacteremia. Because the presence of bacteremia does not necessarily translate into worse outcome, it is questionable whether serum ACE activity would add to a pneumonia severity score focusing on outcome prediction. Furthermore, because serum ACE activity is affected by ACE inhibitors and angiotensin II receptor blockers, as a biomarker, this activity would not suitable for simple and easy scoring. Proteinuria, on the other hand, could be more suitable. Proteinuria is a sign of kidney injury.

When proteinuria is present in a patient presenting in emergency settings, clinicians might, for example, be cautious with nephrotoxic medications, such as gentamicine,[33,34] and nurses can be instructed to measure urinary output, especially in patients with low blood pressure.[35] This can prevent a 'second hit' (a second condition that may harm kidney function) and may prevent kidney failure.[36]

**Adjunctive therapy**

***Is a shorter length of stay due to a faster decrease in CRP?***

In our clinical study on the effect of dexamethasone as adjunctive therapy for CAP, we used the length of hospital stay as one of the primary clinical parameters. While this may seem to be a reliable and objective parameter, length of hospital stay is determined by the application of discharge rules, which in daily clinical practice are difficult to specifically formulate and objectively apply. Patients are ready for discharge when they are clinically stable. However, a consistent decrease in CRP levels may also convince the treating physician that a patient is recovering. Corticosteroids have a direct effect on the transcription of pro-inflammatory cytokine genes. Inhibition of IL-6 leads to decreased production of CRP in the liver. Therefore, treatment with corticosteroids will reduce CRP levels. In our clinical study, one might conclude that patients in the dexamethasone group were discharged one day earlier than those in the placebo group due to the more rapid decrease in CRP levels.

There are good arguments against this reasoning. First, a treating physician sees patients daily on clinical rounds. If CRP is decreasing but a patient still has shortness of breath, fever or any other major complaint, it is unlikely that the patient would be discharged only because of the decreasing CRP. Second, when patients are discharged only based on decreasing CRP level, it would be expected that readmissions, for conditions such as empyema for example, would increase in this group of patients. We did not observe any significant differences in the number of readmissions, but our sample size may have been too small to observe such an effect.

***Dexamethasone treatment for all CAP patients?***

In chapter 10, it was shown that dexamethasone therapy can reduce length of hospital stay in patients with CAP. In a *post-hoc* analysis of this data (*Figure 1, Table 1*), we observed a one day reduction in the length of stay in the patients with PSI class I-III and a three day reduction in the patients with PSI class IV-V. Theoretically, it is expected that patients with more severe inflammation would benefit more from corticosteroid treatment. This led to the study described in chapter 11 in which the cytokine response in patients with CAP and the effect

of dexamethasone on this response were analysed. In the patients with the highest cytokine response, dexamethasone treatment not only reduced length of stay but also reduced mortality and ICU admission (as a combined endpoint). In the literature on corticosteroids and sepsis, it is suggested that not only high cytokine response but also adrenal insufficiency is responsible for increased mortality. Annane *et al*. conducted a series of studies on corticosteroids in patients with sepsis and adrenal insufficiency.[37,38] This adrenal insufficiency was defined as a cortisol level <10 µg/dL or a delta serum cortisol of <9 µg/dL after administration of 250 µg synthetic ACTH.[39] This second criterion (response to corticotropin) has been questioned in several reviews; however, it is commonly used to diagnose adrenal insufficiency.[40,41] Other studies have shown that adrenal insufficiency, as defined by a cortisol level of <10 µg/dL, was not associated with adverse outcome in patients with CAP.[42] Therefore, in chapter 12, cytokine levels were correlated to cortisol levels. Cortisol is needed to control inflammation; consequently, the combination of a high cytokine response but a low cortisol level may indicate a patient with a lack of sufficient cortisol response in CAP. Although this was a *post-hoc* analysis, it was quite illustrative that this subgroup of patients with a high cytokine response but a low cortisol level benefited most from corticosteroid therapy.

Another finding from these last two studies was that in patients with only moderate cytokine responses, ICU admission and mortality were higher, but not significantly higher, in the dexamethasone group (7.5% vs. 3.6%; p=0.47). It might be possible that in patients with a 'low' or 'appropriate' cytokine response, treatment with dexamethasone results in too much suppression of the inflammation response. In addition to cytokine response and cortisol levels, other factors, such as age, causative agent and comorbidities, are likely to be of importance in the effect of corticosteroids in CAP.

These findings raise the question of which patients would benefit most from dexamethasone therapy. According to the data presented in chapter 10, overall, patients with CAP benefit from corticosteroids in terms of length of hospital stay. *Post-hoc* data showed that patients with 'high' pro-inflammatory cytokine responses were likely to benefit more from corticosteroid therapy. When this 'excessive' cytokine response was accompanied by a relatively low cortisol level, the beneficial effect might be even stronger. The last two findings were *post-hoc* and the study was not powered on these hypotheses. The planned follow-up study, which will be multi-centred and include more patients, may confirm and elaborate on this preliminary conclusion.

Future research concerning the role of dexamethasone as adjunctive therapy in CAP will have to clarify those uncertainties. First, our results need to be confirmed in a large multicentre RCT, preferably with patients stratified by PSI

class. This study needs to be sufficiently powered to analyse the beneficial effect of dexamethasone in subclasses, such as patients with high cytokine responses and those with discrepancies between cortisol levels and cytokine responses. Second, as mentioned above, adjunctive treatment with dexamethasone may not be beneficial for all patients. A large study is also needed to reveal patients with unfavourable effects of dexamethasone treatment and to better evaluate potential harm due to dexamethasone (e.g., development of empyema or readmission). Such a follow-up study should also offer the possibility to address a number of additional issues such as the following: the effect of intravenous (iv) versus oral dexamethasone, which might prevent unnecessary iv treatment; prolonged treatment with corticosteroids to overcome a rebound effect of cytokine response; the correlation between quality of life score on day 30 with cytokines and cortisol levels to more fully understand a prolonged recovery from an episode of CAP; and the effect of dexamethasone on persistent pulmonary or renal injury.

**Table 1.** Length of stay, subdivided by pneumonia severity score.

| Length of hospital stay | Dexamethasone Group n=151 | Placebo Group n=153 | p |
|---|---|---|---|
| PSI Class I, II, III, days | 5.5 (4.5-7.5) | 7.0 (4.5-8.5) | 0.051 |
| PSI Class IV & V, days | 7.5 (6.5-10.5) | 10.5 (7.5-13.5) | 0.023 |

Data are presented as median (interquartile range)

***What are other low-cost adjunctive therapies for CAP?***

In addition to the effect of dexamethasone on CAP, other relatively low cost interventions have been suggested to optimise current treatment strategies. There is increasing discussion about a potential role for macrolides and statins in the management of CAP.[43-45] Statins have been shown to have significant anti-inflammatory properties and may modify the balance of coagulation towards a less prothrombotic state. The majority of the studies on this subject have suggested that statins have a beneficial effect on the outcome of infection; however, due to their observational design, firm conclusions cannot be drawn.[46] Therefore, the clinical benefit of statin therapy in sepsis remains to be determined by an RCT. Macrolides are also known to possess immunomodulatory effects in patients with CAP.[47,48] These molecules have direct antimicrobial activity by stimulating the host defence against bacteria via stimulation of leukocyte degranulation, phagocytosis and oxidative burst, and after the acute infection, neutrophils that are primed by cytokines or LPS

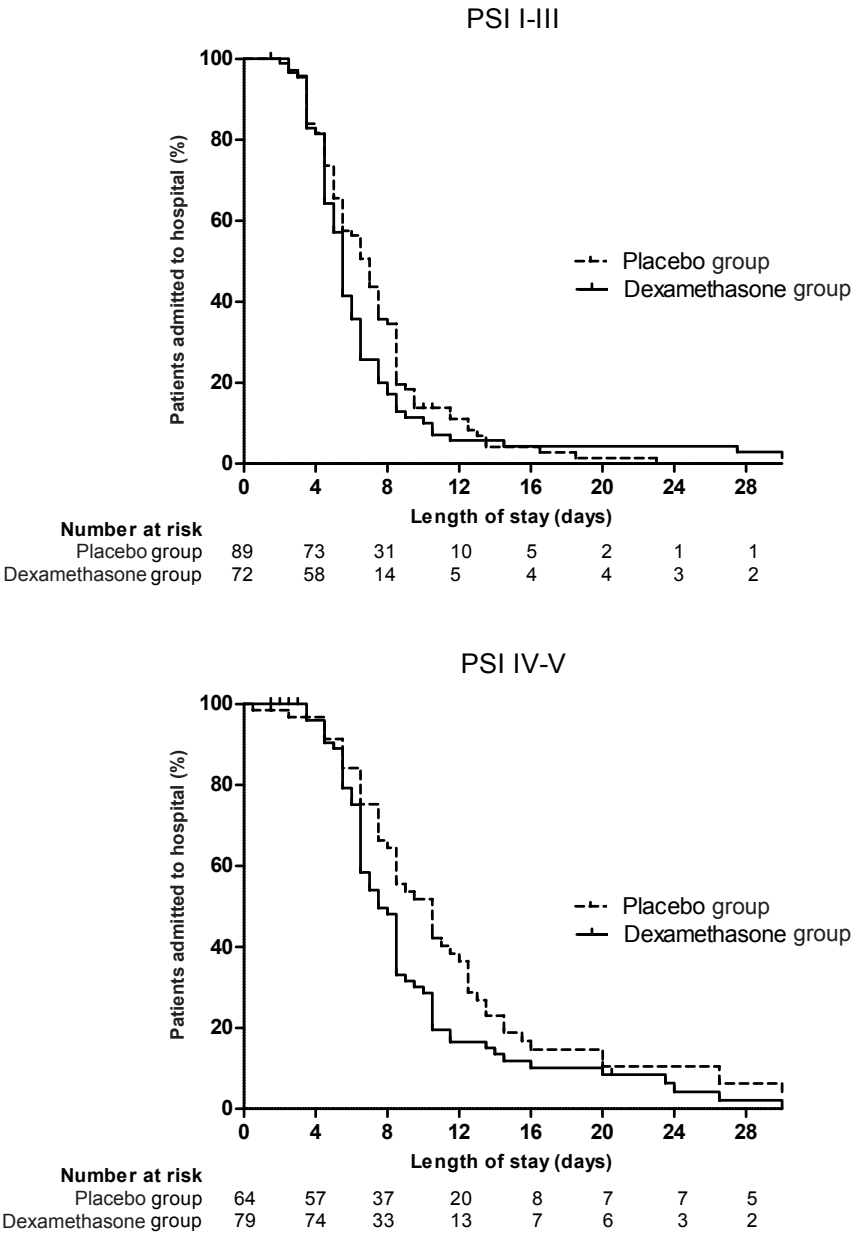


**Figure 1.** Kaplan-Meier analyses of the effect of dexamethasone on length of hospital stay, subdivided by the pneumonia severity score.

are inhibited by macrolides, resulting in the resolution of the inflammatory response. Moreover, macrolides reduce bacterial load with less cell-wall lysis compared with beta-lactam antibiotics, which result in more gradual reduction in bacterial load and therefore, more gradual release of immunologically reactive components, which in turn may prevent an extended systemic inflammatory response. However, studies on the beneficial effects of macrolides are inconsistent, and for this form of adjunctive therapy, RCTs are needed.[49,50]

## Conclusions

CAP is not a single disease. There are several causative agents, and the clinical features of patients (e.g., age, comorbidities, genetic profiles of the immune response) differ widely. Therefore, the ultimate goal is antibiotic treatment tailored for the causative agent and personalised adjunctive therapy. However, the major problem is that because of the heterogeneity of the immune system of the host and the causative pathogens, it is virtually impossible to determine the level of immune response required during CAP in a single patient. Appropriate functioning of the immune system of a patient during an infection would ideally be assessed during an infection-free period. Determination of the genetic polymorphisms in innate immunity genes theoretically could predict the nature of the cytokine response. Our data in chapter 3 indicate that the current level of understanding of genetic polymorphisms is insufficient to predict the level of the innate immune response following natural infection. Vaccination, as a method to determine the immune status, is not informative because it largely lacks the inflammatory compounds of the response. Because of these arguments, it is currently impossible to conclude for the individual patient whether the observed response of the (innate) immune system during an infection is deficient, sufficient, or even overdone. In this thesis, as a first step to personalised adjunctive therapy in CAP, a combination of clinical and biological characteristics has been applied, allowing the classification of patients into different categories of immune response. Future research has to clarify whether this classification can optimise the administration of adjunctive therapy to improve the clinical management of CAP.

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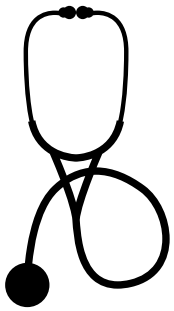


## Introductie

**Wat is een longontsteking?**

Een longontsteking is een ontsteking van het longparenchym, veroorzaakt door een micro-organisme, meestal een bacterie of (minder vaak) een virus. Bij patiënten zonder immuun stoornissen is *Streptococcus pneumoniae* de meest voorkomende verwekker, gevolgd door *Haemophilus influenzae*, *Legionella* soorten en virussen. Een longontsteking wordt gekenmerkt door een ontsteking in de long zelf waardoor de patiënt hoest, sputum opgeeft, zich benauwd voelt en pijn heeft op de borst. De systemische ontstekingsrespons zorgt ervoor dat er symptomen ontstaan zoals koorts of hypothermie, transpireren en rillingen. De thoraxfoto toont infiltraten en in het bloed wordt vaak een verhoogd aantal leukocyten en een verhoogd CRP gevonden.

Een longontsteking kan worden ingedeeld naar 'buiten het ziekenhuis opgelopen' (community-acquired), 'in het ziekenhuis opgelopen' (hospital-aquired) of 'opgelopen terwijl de patiënt aan de beademing ligt' (ventilator-associated). Dit proefschrift gaat over de buiten het ziekenhuis opgelopen longontsteking. Dit soort longontsteking is wereldwijd een van de belangrijkste oorzaken voor ziekenhuisopname en de op twee na meest voorkomende oorzaak van overlijden. Ook in Nederland komt een longontsteking vaak voor, rond de 8,3 per 1000 mannen en 8,4 per 1000 vrouwen per jaar. Dat komt neer op zo'n 135.000 ziekte episodes per jaar. Met name ouderen en kinderen lopen een groter risico op het krijgen van een longontsteking. Ongeveer 22.000 patiënten moeten worden opgenomen en 5.500 patiënten overlijden aan de longontsteking. De opnames in het ziekenhuis voor een longontsteking gaan gepaard met hoge kosten voor de gezondheidszorg.

## Aanleiding en doel van het onderzoek

De behandeling van een longontsteking wordt de laatste jaren bemoeilijkt door resistentie van bacteriën tegen antibiotica, een steeds ouder wordende bevolking en een toename van het aantal patiënten met een immuun stoornis (bijvoorbeeld door chemotherapie). Naast deze moeilijkheden werd Nederland ook nog eens getroffen door de Q koorts epidemie in 2009 en 2010 en de Mexicaanse griep in de herfst van 2010.

De gangbare behandeling voor een longontsteking is vroege herkenning en het geven van de juiste antibiotica, het liefst binnen 4 uur nadat de longontsteking is vastgesteld. Ondanks dat er de laatste jaren nieuwe vaccins, antibiotica en microbiologische testen zijn ontwikkeld wordt een longontsteking nog steeds gekenmerkt door een hoge ziektelast, een niet te onderschatten overlijdensrisico en significante kosten voor de gemeenschap.

In dit proefschrift hebben we gezocht naar mogelijkheden om de kwaliteit van de zorg rondom een longontsteking te verbeteren. De mogelijkheden die werden onderzocht lagen op het terrein van preventie, een beter begrip van de immuun respons tijdens een longontsteking, het ontwikkelen van biomarkers en extra behandelmogelijkheden naast de gangbare antibiotica.

## Strategieën om de behandeling van een buiten het ziekenhuis opgelopen longontsteking te verbeteren.

### Gevoeligheid voor en preventie van longontsteking

Sinds het begin van de 20e eeuw hebben toegenomen hygiënische maatregelen en het ontwikkelen van sanitair ervoor gezorgd dat de incidentie van infecties, waaronder longontsteking, fors afnamen. Het invoeren van vaccinatie tegen influenza en het recent geïntroduceerde vaccineren van kinderen tegen *S. pneumoniae* hebben ook geleid tot een afname van infecties. Verder is de afgelopen jaren gebleken dat snel ingrijpen bij epidemieën (Q koorts en H1N1 ("nieuwe griep")) de incidentie van longontstekingen kan beïnvloeden. Onhygiënische omstandigheden en epidemieën zijn een risico voor het krijgen van een longontsteking. Echter, in de afgelopen jaren zijn er verschillende studies verschenen waarin werd gesuggereerd dat een bepaald soort maagzuuronderdrukkende medicijnen, de proton pomp remmers (PPIs), ook het risico op een longontsteking verhogen. In **hoofdstuk 2** werden daarom patiënten met een longontsteking vergeleken met gezonde controles. De controle patiënten werden verkregen uit de PHARMO database, een database waarin het medicijngebruik van een grote groep Nederlanders wordt bijgehouden. Voor iedere patiënt met een longontsteking werden vier gezonde controles gezocht. De patiënten werden met elkaar vergeleken en er werd aangetoond dat het risico op een longontsteking inderdaad verhoogd was tijdens het gebruik van PPIs (Odds Ratio (OR) 1,6 [95% confidence interval (CI) 1,2 – 2,2]). Er werd ook bevestigd dat het risico het hoogst was binnen 15 dagen na de start van de behandeling (OR 3,1 [95% CI 1,1 – 8,8]). Omdat het tegenstrijdig lijkt dat het risico hoger is naarmate de patiënt korter PPIs gebruikt, zijn de patiënten die recent gestart waren met PPIs en een longontsteking kregen verder onderzocht. In de huidige literatuur worden vaak protopathic bias (een patiënt krijgt een PPI voor de eerste verschijnselen van een longontsteking) of een veranderde microbiële flora in de maag genoemd als oorzaken voor dit verschijnsel. Uit ons onderzoek bleek dat protopathic bias niet de verklaring kon zijn voor dit effect. Verder konden we aantonen dat deze patiënten geen ander soort ziektemakend micro-organisme (bv een micro-organisme dat gastro-intestinaal voorkomt) hadden dan de andere patiënten met een longontsteking.

## De immuun respons tijdens een longontsteking

Het menselijk lichaam kan zich kan zich op 3 manieren verdedigen tegen het binnendringen van lichaamsvreemd materiaal: de mechanische afweer, het aangeboren immuunsysteem en het verworven immuunsysteem. In de long bestaat de mechanische afweer uit trilhaar bewegingen, hoesten en niesen. De aangeboren afweer bestaat uit fagocyten (witte bloedcellen) die geactiveerd worden door cytokines (ontstekingsstoffen) en complement (eiwitten die binden aan bacteriën zodat ze daarna gemakkelijker opgeruimd kunnen worden). De aangeboren afweer is altijd direct paraat in tegenstelling tot de verworven afweer dat er, na contact met een pathogeen, dagen tot weken over doet om op gang te komen. De verworven afweer is echter zeer specifiek en heeft geheugen, waardoor bij een volgend contact met een bekend pathogeen dit snel opgeruimd kan worden.

De genen die coderen voor eiwitten van het aangeboren immuun systeem bevatten vele inter-individuele variaties (polymorfismen en haplotypes genoemd). In de afgelopen jaren zijn variaties geïdentificeerd die geassocieerd zijn met een verhoogd risico op het ontwikkelen van een longontsteking of met de ernst van een longontsteking. Bijvoorbeeld het polymorfisme op het gen van cytokine interleukine (IL)-6, -174C/C, is geassocieerd met een verhoogde kans op het krijgen van een longontsteking doordat het zorgt voor een verlaagde ontstekingsrespons. Ook in het complement systeem zijn polymorfismen bekend. Het polymorfisme +6359C>T op het ficoline-2 gen leidt tot een aminozuur verandering die ervoor zorgt dat ficoline-2 minder goed bindt aan zijn ligand op de bacterie. Deze bevindingen suggereren dat er ook een genetisch basis is voor de kans op een longontsteking en de ernst van een longontsteking.

In **hoofdstuk 3** werd de cytokine respons onderzocht in een cohort van 201 niet immuun-gecompromitteerde patiënten met een longontsteking. IL-6 en IL-8, samen met IL-1Ra en IL-10 zijn acute fase eiwitten tijdens een longontsteking. IL-6 is een betere biomarker voor het beloop van een longontsteking dan het veel in de kliniek gebruikte CRP doordat het sneller reageert op behandeling. Ook het effect van polymorfismen en haplotypes op de cytokine respons werd onderzocht. In onze data, beïnvloedde polymorfismen en haplotypes in de IL-6, IL-8 en IL-10 genen niet de hoeveelheid cytokines die geproduceerd werden en hadden deze polymorfismen geen invloed op het beloop van de longontsteking. Verder werd gevonden dat de systemische cytokine respons wel wordt beïnvloed door het soort ziekte-makende micro-organisme. Patiënten met een longontsteking door pneumokokken hadden een significant

hoger IL-6 en IL-1Ra dan patiënten met een longontsteking veroorzaakt door een ander micro-organisme. In een niet-gerandomiseerde setting toonden we aan dat de cytokine respons tijdens een longontsteking onderdrukt wordt door het gebruik van corticosteroïden.

Naast de cytokines respons, zoals bestudeerd in hoofdstuk 3, is ook het complement systeem belangrijk in de aangeboren afweer. Het is bekend dat mannose bindend lectine (MBL) en ficoline-2 belangrijke delen van het complement systeem zijn en dat ze beide goed aan de bacterie *Staphylococcus aureus* binden. In **hoofdstuk 4** werd de rol van polymorfismen in de genen van deze twee eiwitten onderzocht bij patiënten op continue ambulatoire peritoneaal dialyse (CAPD) met een *S. aureus* peritonitis (buikvliesontsteking). In het cohort van 105 CAPD patiënten leidde het polymorfisme +6359C>T tot een verhoogd risico op een *S. aureus* peritonitis. Patiënten met het genotype +6359T/T hadden vaker een peritonitis dan de patiënten met het normale genotype +6359C/C (OR 5,57 [95% CI 1,29 – 24,05]). Polymorfismen in het MBL-gen waren niet geassocieerd met een verhoogd risico op het krijgen van een *S. aureus* peritonitis.

Uit eerdere literatuur is bekend dat ficoline-2 tekort is geassocieerd met infectieuze longziekten bij kinderen. Dit leidde tot de studie in **hoofdstuk 5**, waarin onderzocht werd of MBL, ficoline-2 en vervolgens MASP-2 (volgende stap in de complement cascade) binden op de *S. pneumoniae* bacterie. MASP-2 binding werd gevonden in 19/32 patiënten (59%), MBL binding in 13/32 patiënten (41%) en ficoline-2 binding in 28/32 patiënten (88%). In een subgroep van patiënten waarin wel binding van MBL werd gezien, maar geen ficoline-2 binding, werd ook geen MASP-2 binding gevonden. MASP-2 binding bleek verder onafhankelijk van het serotype van de pneumokokken. In deze studie werd aangetoond dat pneumokokken MASP-2 kunnen binden en dat het waarschijnlijk voornamelijk via binding aan ficoline-2 gaat.

Niet alleen een beter begrip van de aangeboren afweer maar ook nieuwe inzichten in het verworven immuunsysteem kunnen leiden tot verbetering in de zorg rondom een longontsteking. In **hoofdstuk 6** werden serotype-specifieke antilichaam concentraties gemeten op verschillende tijdstippen na het ontstaan van de longontsteking met een kwantitatieve multiplex immunoassay. Deze assay kan 14 verschillende serotype-specifieke anti-kapsel polysaccharide IgG antilichamen aantonen. Met deze nieuwe methode werd aangetoond dat het geschatte aantal longontstekingen veroorzaakt door *S. pneumoniae* bij patiënten waarvan de verwekker niet bekend was rond de

57% (95%CI 36 – 86%) ligt. Deze methode is niet geschikt voor gebruik in de dagelijkse praktijk, maar deze bevinding verantwoordt het gebruik van alleen beta-lactam therapie bij de meeste patiënten met een longontsteking. Verder kan het bijdragen aan de discussie over vaccinatie van risicopatiënten met het 23-valente polysaccharide vaccin.

## Biomarkers

Een biomarker is in het algemeen een stof die objectief gemeten en geëvalueerd kan worden als een indicator voor een biologisch proces, tijdens gezondheid of tijdens ziekte of als respons op therapie. Deze biomarkers kunnen ook van nut zijn tijdens de behandeling van een longontsteking. In dit proefschrift werden twee mogelijke biomarkers onderzocht om de ernst en het beloop van een longontsteking te kunnen voorspellen.

In **hoofdstuk 7** werd de bijdrage van serum angiotensin I – converting enzyme (ACE) activiteit ten tijde van de opname in het ziekenhuis als prognostische marker onderzocht. Serum ACE wordt met name aangemaakt in de microbloedvaten van de long en is al bij verschillende longaandoeningen bestudeerd. ACE is bekend doordat het een belangrijke rol speelt tijdens de bloeddruk regulatie. ACE zorgt voor de omzetting van angiotensine I in angiotensin II, een krachtige vasoconstrictor en stimulator van aldosteron waardoor de bloeddruk stijgt. In een eerdere studie bij patiënten met een longontsteking werd gevonden dat patiënten met een laag ACE gehalte in het bloed een slechtere prognose hadden van hun longontsteking. Maar omdat het hier ging om een te kleine groep patiënten kon deze bevinding niet hard gemaakt worden. Daarom werd in het onderzoek van hoofdstuk 7 een tweede cohort patiënten toegevoegd aan het eerste cohort. De sterke daling van ACE activiteit tijdens een longontsteking werd hierin bevestigd, maar de hoogte van ACE bleek niet de prognose van een longontsteking te kunnen voorspellen. Interessant genoeg was een ACE activiteit lager dan 24 U/L tijdens opname wel een sterke voorspeller voor het hebben van een bacteriëmie (OR 3,93 [95% CI 1,57 – 9,87]). Wanneer de ACE activiteit gecombineerd wordt met het CRP gehalte en het leukocyten aantal, kan het de kans op bacteriëmie voorspellen voordat de bloedkweken bekend zijn.

In **hoofdstuk 8** wordt de incidentie en de voorspellende waarde van proteïnurie (eiwit in de urine) als biomarker voor het beloop van een longontsteking bestudeerd. Recente literatuur heeft laten zien dat proteïnurie bij patiënten met chronische nierziekten, maar ook in de algemene bevolking, een voorspeller is van overlijden. Dit is onafhankelijk van de nierfunctie. De voorspellende

waarde van proteïnurie tijdens een acute ziekte episode is nooit onderzocht. Proteïnurie kan een vroege maat voor nierschade zijn en van nierschade is bekend dat het ongunstig is voor het beloop van de longontsteking. Bij 319 van de 496 patiënten met een longontsteking die werden onderzocht (64%), is er urine afgenomen en is deze gecontroleerd op de dag van opname. Tweeënzestig procent van de patiënten bleek proteïnurie te hebben. De nu gangbare maat voor acuut nierfalen zijn de RIFLE criteria. We onderzochten in deze studie of proteïnurie een toegevoegde waarde zou kunnen hebben naast of in combinatie met de RIFLE criteria. Proteïnurie tijdens opname was (in tegenstelling tot de RIFLE criteria) geassocieerd met een langere opnameduur. Zowel proteïnurie als de RIFLE criteria waren geen onafhankelijke voorspeller voor de ziekenhuismortaliteit of de 1-jaars mortaliteit.

## Additionele therapie voor een longontsteking

Op dit moment is de belangrijkste behandeling voor een longontsteking het zo snel mogelijk toedienen van antibiotica. Echter, de meest voorkomende bacteriën laten steeds vaker resistentie zien tegen antibiotica. Het is ook bekend dat de ernst van een longontsteking mede wordt bepaald door de hoeveelheid ontstekingscellen die worden gevormd. Daarom is het interessant (en in de toekomst misschien zelfs noodzakelijk) om te zoeken naar additionele (niet-antibiotische) ontstekingsremmende medicatie voor patiënten met een longontsteking.

In **hoofdstuk 9** wordt de literatuur over de verschillende opties voor additionele therapie op een rij gezet en beoordeeld. Indien de antibiotica resistentie doorzet, kan het zijn dat deze aanvullende therapie, in de toekomst, zelfs de enige optie is. Gezien de huidige bewijsvoering kunnen zowel corticosteroïden, als geactiveerd proteïne C (APC), als immuunglobulines niet gebruikt worden zonder toevoeging van antibiotica. MBL en Toll-like receptor (TLR) agonisten en antagonisten zijn aantrekkelijke opties maar bewijsvoering voor een echte toegevoegde waarde ontbreekt nog. Corticosteroïden zijn beschikbaar en hebben hun toegevoegde waarde bewezen als aanvullende therapie in grote studies bij mensen met een sepsis of septische shock. Dit, in tegenstelling tot het gebruik van corticosteroïden bij patiënten met een longontsteking. De studies hierover zijn klein, niet gerandomiseerd en wisselend van uitkomst.
Daarom werd in **hoofdstuk 10** een gerandomiseerde, placebo-gecontroleerde studie uitgevoerd. Hierin werd onderzocht wat het effect is van het toevoegen van een corticosteroid (dexamethason) aan antibiotica op de opnameduur in het ziekenhuis tijdens een longontsteking. Opgenomen, niet immuun-gecompromitteerde patiënten werden willekeurig toegewezen aan een

interventie groep of een placebo groep. De interventie groep kreeg gedurende vier dagen 5 mg dexamethasone eenmaal per dag. De placebo groep kreeg op deze tijdstippen steriel water.
Er werden 304 patiënten geïncludeerd (153 in de placebo groep en 151 in de interventie groep). De mediane opnameduur was 6,5 dagen voor de patiënten in de dexamethason groep en 7,5 dagen voor de placebo groep (p=0,048). Patiënten in de dexamethason groep lieten een snellere daling van het CRP en IL-6 gehalte zien dan de patiënten in de placebo groep. De kwaliteit van leven met betrekking tot het sociaal functioneren was, in de dexamethason groep, op dag 30 zo'n 15% beter. Patiënten in deze groep hadden vaker last van hyperglycemie, maar ernstige bijwerkingen waren zeldzaam en de frequentie verschilde niet in beide groepen. Een *post hoc* analyse (waarvan de data niet vermeld zijn in dit proefschrift) toonde aan dat het effect van dexamethason groter is bij patiënten met een longontsteking van pneumonia severity index (PSI) klasse IV of V. In deze groep is de mediane reductie in opnameduur zelfs 3 dagen (7,5 vs. 10,5 dagen, p=0,023).

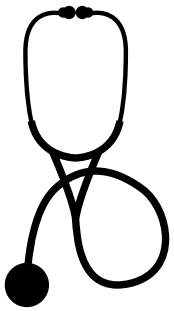
Om verder uit te zoeken welke patiënten baat hebben bij extra therapie met dexamethason, werd in **hoofdstuk 11** de cytokine respons van de 304 patiënten onderzocht. De patiënten met een longontsteking die behandeld werden met extra dexamethason hadden een snellere daling van hun cytokine waarden. Er werd een groep patiënten samengesteld waarin de cytokines IL-6, IL-8 en MCP alle drie verhoogd waren (IL-6 (≥4,53 mg/dL), IL-8 (≥3,79 mg/dL) en MCP (≥6,70 mg/dL). Twee van de 24 patiënten uit deze groep (8,3%) die dexamethason kregen overleden of hadden IC opname nodig tegenover 8/17 patiënten in de placebo groep (47%) (p<0,01, OR 0,10 [95%CI 0,02 – 0,58]).

Cortisol is een anti-inflammatoir hormoon dat aangemaakt wordt in de bijnierschors. Het remt de pro-inflammatoire cytokines en andere ontstekings mediatoren. Omdat cortisol dus nodig is om de ontstekingsreactie onder controle te houden, wordt een inadequate cortisol respons geassocieerd met een slechter beloop van de longontsteking. Bij kritisch zieke patiënten is een inadequate bijnierschors respons (gedefinieerd als een cortisol <10 µg/dl of een delta cortisol <9 µg/dL na een gift van 250 µg synthetisch ACTH) geassocieerd met een verhoogd risico op overlijden. Dit wordt ook wel "critical-illness related corticosteroid insufficiency" (CIRCI) genoemd. In de huidige literatuur wordt gesuggereerd dat patiënten met zo'n bijnierschors insufficiëntie het meeste baat hebben van corticosteroid therapie.
In **hoofdstuk 12** wordt aangetoond dat voor patiënten met een longontsteking een cortisol <10 µg/dl niet geassocieerd is met een slechter beloop. Patiënten

met een cortisol <10 µg/dl hebben een milde longontsteking met lage cytokine waarden in plaats van bijnierschors insufficiëntie. De patiënten die wel baat hebben bij dexamethasone therapie zijn de patiënten met hoge cytokine waarden en daarbij een discrepant laag cortisol level (laagste 50% van de patiënten met een hoge cytokine respons). In deze groep hadden de dexamethason gebruikers geen IC opname nodig en overleed niemand, terwijl dit wel het geval was bij 31% van de patiënten in de placebo groep. Daarom wordt in dit hoofdstuk gesteld dat het deel van de definitie van CIRCI, namelijk het hebben van een cortisol <10 µg/dl, niet geschikt is voor patiënten met een longontsteking. Verder blijkt dat dexamethason IC opname en overlijden reduceert bij de patiënten met een hoge cytokine respons en een discrepant laag cortisol. De correlatie van cortisol gehalte aan de cytokine respons geeft mogelijk het beste aan welke patiënten echt baat hebben bij dexamethason therapie.

## Conclusies en aanbevelingen

De studies uit dit proefschrift geven inzicht in de mogelijke strategieën om de zorg rondom een longontsteking te verbeteren. Echter, er zijn nog veel vragen niet beantwoord. In **hoofdstuk 13** wordt besproken of een longontsteking voorkomen kan worden door patiënten te wijzen op de risico's van PPI gebruik en op een heel andere manier, door patiënten te vaccineren. Ten aanzien van de immuunrespons tijdens een longontsteking kan gesteld worden dat complement waarschijnlijk wel een rol speelt bij de afweer tegen *S. pneumoniae* maar dat het voorlopig niet duidelijk is wat de precieze betekenis hiervan is voor de patiënt en of deze patiënten bijvoorbeeld ook gevaccineerd zouden moeten worden. Verder wordt in het hoofdstuk bediscussieerd of de nieuwe biomarkers de huidige score om de ernst van een longontsteking vast te stellen (de PSI score) zouden kunnen verbeteren. ACE kan een bijdrage leveren aan het voorspellen van een bacteriëmie, maar proteïnurie kan mogelijk meer belangrijke uitkomstmaten, zoals overlijden, voorspellen.
Over additionele therapieën tijdens een longontsteking wordt gesteld dat verder onderzoek helderheid moeten verschaffen over de rol van dexamethason tijdens een longontsteking. Hebben alle patiënten er baat bij, of alleen de patiënten met een ernstige longontsteking, of alleen met hoge cytokine waarden? En speelt bijnierschors insufficiëntie een rol bij de aanvullende waarde van dexamethason? Verder zijn er naast corticosteroïden nog andere, additionele therapieën beschikbaar waar verder onderzoek naar gedaan kan worden.



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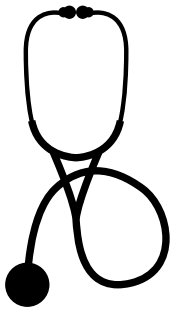


## List of Publications

Meijvis SCA, Cornips MCA, Endeman H, Ruven HJT, Danser AH, Biesma DH, Leufkens HGM, Bos WJW, van de Garde EM.
Prognostic value of serum angiotensin-convertingenzyme activity for outcome of community-acquired pneumonia.
*Clin Chem Lab Med. 2011 Sep;49(9):1525-32*

Meijvis SCA, Hardeman H, Remmelts HHF, Heijligenberg R, Rijkers GT, van Velzen-Blad H, Voorn GP, van de Garde EM, Endeman H, Grutters JC, Bos WJW, Biesma DH.
Dexamethasone and length of hospital stay in patients with community-acquired pneumonia: a randomised, double-blind, placebo-controlled trial.
*Lancet. 2011 Jun 11;377(9782):2023-30.*

Meijvis SCA, Cornips MCA, Voorn GP, Souverein PC, Endeman H, Biesma DH, Leufkens HGM, van de Garde EM.
Microbial evaluation of proton pump inhibitors and the risk of pneumonia.
*Eur Respir J. 2011 Nov;38(5):1165-72*

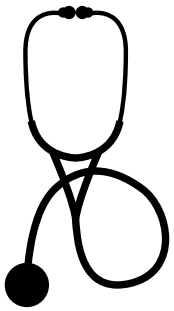
van Mens SP, Meijvis SCA, Endeman H, van Velzen-Blad H, Biesma DH, Grutters JC, Vlaminckx BJM, Rijkers GT.
Longitudinal analysis of pneumococcal antibodies during community-acquired pneumonia reveals a much higher involvement of Streptococcus pneumoniae than estimated by conventional methods alone.
*Clin Vaccine Immunol. 2011 May;18(5):796-801.*

Meijvis SCA, Grutters JC, Thijsen SF, Rijkers GT, Biesma DH, Endeman H.
Therapy in pneumonia: what is beyond antibiotics?
*Neth J Med. 2011 Jan;69(1):21-6.*

Meijvis SCA, Endeman H, Rijkers GT, van Velzen-Blad H, van Moorsel CH, Grutters JC, Biesma DH.
Systemic cytokine response in patients with community-acquired pneumonia.
*Eur Respir J. 2011 Jun;37(6):1431-8.*

Meijvis SCA, Herpers BL, Endeman H, de Jong B, van Hannen E, van Velzen-Blad H, Krediet RT, Struijk DG, Biesma DH, Bos WJ.
Mannose-binding lectin (MBL2) and ficolin-2 (FCN2) polymorphisms in patients on peritoneal dialysis with staphylococcal peritonitis.
*Nephrol Dial Transplant. 2011 Mar;26(3):1042-5.*

Meijvis SCA, Endeman H, Geers ABM, ter Borg EJ. Extremely high serum ferritin levels as diagnostic tool in adult-onset Still's disease.
*Neth J Med. 2007 Jun;65(6):212-4.*



De afgelopen 4 jaar heb ik met zeer veel plezier gewerkt aan mijn proefschrift. Promoveren doe je niet alleen. Ik wil iedereen die heeft meegewerkt aan de totstandkoming van mijn proefschrift, en in het bijzonder aan de Ovidius studie, zeer hartelijk bedanken. Een aantal mensen wil ik speciaal noemen.

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Mijn copromotoren, dr. ir. G.T. Rijkers en dr. W.J.W. Bos.
Beste Ger, ik kan niet anders zeggen dan dat je tijdens mijn gehele promotie mijn steun en toeverlaat bent geweest. Je was altijd kritisch, maar hielp me vervolgens ook verder. Je hebt me zelfs een klein beetje liefde voor hardcore labwerk bijgebracht! Zeer veel dank voor je eindeloze geduld en altijd snelle reactie op al mijn vragen.
Beste Willem Jan, hoewel je pas later bij mijn pneumonie onderzoek betrokken raakte, heb ik door de Thales studie wel de grondbeginselen van onderzoek doen geleerd. Het werd zelfs mijn eerste echte publicatie. Dank voor je inspirerende en enthousiaste hulp.

Prof.dr. J.W.J. Lammers, prof.dr. I.M. Hoepelman, prof.dr. M.M.E. Schneider, prof. dr. H.G.M. Leufkens en prof.dr. T. van der Poll bedankt voor het beoordelen van mijn manuscript.

Hans Hardeman, mijn mede-onderzoeker.
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Hilde Remmelts, onderzoekster in Ede en mede-promovenda.
Beste Hilde, nadat Winnifred Staartjes de Ovidius studie in Ede had opgezet heb jij met zeer veel toewijding en enthousiasme meer dan 60 patiënten geincludeerd. In de loop van de tijd ontstond het plan dat jij ook zou promoveren op de data die we samen verzameld hadden. Daar ben ik zeer blij om. Zonder jou was mijn onderzoeksjaar zeker niet zo leuk geweest. Veel succes met je eigen proefschrift.

Claire Cornips, apotheker en schoonzus.
Beste Claire, wat was het gezellig dat je bij mij en Ewoudt onderzoek kwam doen! Je hebt hard gewerkt en me zeer veel werk uit handen genomen. Leuk dat je ook dokter wordt. Ik hoop dat je zelf ooit gaat promoveren want je bent er voor in de wieg gelegd.

Simone Spoorenberg, student geneeskunde.
Beste Simone, het was een groot genoegen om samen met je een artikel te schrijven. Je hebt hard gewerkt en ik wens je heel veel succes bij de vervolgstudie naar dexamethasone bij CAP.

Rik Endeman, internist-intensivist te Amsterdam.
Beste Rik, jij hebt mij als eerste enthousiast gemaakt voor wetenschappelijk onderzoek en me begeleid bij mijn eerste voorzichtige pogingen iets op te schrijven. Samen met Douwe heb je aan de wieg gestaan van dit proefschrift. Ik ben zeer blij dat ons gezamenlijke artikel de basis vormt voor dit proefschrift en dat je altijd bereid was mee te denken over stijgende en dalende cytokines.

Maatschap Interne Geneeskunde, in het bijzonder dr. A.B.M. Geers, opleider.
Zeer veel dank voor het mogelijk maken van mijn studie, het faciliteren van mijn onderzoeksjaar en de cursus statistiek in Rome. Daarnaast ben ik zeer blij dat ik door zo'n fijne maatschap word opgeleid.

Maatschap Medische Microbiologie en Immunologie
Dank voor het ondersteunen van mijn onderzoek en het feit dat ik een jaar zeer gastvrij gebruik mocht maken van jullie arts-assistentenkamer. Jullie enthousiasme over mijn onderzoek was hartverwarmend. In het bijzonder wil ik Heleen van Velzen-Blad en Paul Voorn bedanken voor jullie kritische beoordeling van onderzoeksplannen en manuscripten en daarnaast de belangstelling voor wat er in de rest van mijn leven gebeurde.

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Ewoudt van de Garde, ziekenhuisapotheker.
Beste Ewoudt, naast hulp bij praktische zaken (zoals het schuren van de ampullen) was je ook een zeer gewaardeerde vraagbaak voor statistische vragen. Met het mogelijk maken van het onderzoek van Claire en je uitgebreide revisie van mijn manuscripten heb je een belangrijke bijdrage geleverd aan dit proefschrift.

Rik Heiligenberg, internist te Ede.
Beste Rik, je was meteen enthousiast toen we je benaderde om mee te doen aan de Ovidius studie. Zeer veel dank voor het mogelijk maken van de studie in Ede.

Arts-assistenten Interne Geneeskunde en Longgeneeskunde van het St. Antonius ziekenhuis en de Gelderse Vallei in Ede. Dank voor het includeren van patiënten. Zonder jullie gezelligheid en steun was het nooit gelukt.

Verpleegkundigen en secretaresses van de interne- en longafdelingen, spoedeisende hulp en longfunctie. De Ovidius studie betekende (veel) extra werk voor jullie, maar altijd werd er wel een gaatje gevonden om kweken af te nemen of een buisje bloed te prikken. Dank!

Dr. C.S.P.M. Uiterwaal, klinisch epidemioloog van het Julius Centrum te Utercht.
Beste Cuno, je hebt geholpen bij een klein maar cruciaal gedeelte van mijn proefschrift. Ik ben je daar heel erg dankbaar voor.

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Paranimfen, Irene Lips, radiotherapeut i.o. en Valerie Meijvis, apotheker.
Lieve Irene, ik ben zo blij dat je mijn paranimf bent! Want dan staat er niet iemand naast me die precies snapt wat die cytokines doen tijdens een pneumonie, maar gewoon m'n beste vriendinnetje.
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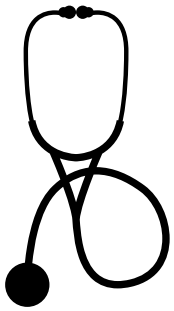
Lieve Pascal, ik kon me niet voorstellen dat er nog een mannetje zou komen waarvan ik zoveel kan houden als van jou. Laten we volop van Hugo genieten!



Sabine Cécile Antoinette Meijvis werd geboren op 5 april 1982 in Breda. Na een jeugd vol met hockey, haalde zij in 2000 haar gymnasium diploma aan het Onze Lieve Vrouwe Lyceum. Aansluitend startte zij haar studie Geneeskunde in Utrecht. Tijdens haar studie liep ze twee keuzecoschappen in Afrika. Een combinatie van gynaecologie en kindergeneeskunde in Mangochi, Malawi en interne geneeskunde in Mwanza, Tanzania. Daarna deed ze wetenschappelijk onderzoek op de afdeling Maag- darm- leverziekten van het UMC Utrecht.

In 2006 liep ze haar laatste keuzecoschap op de afdeling Interne Geneeskunde van het St. Antonius ziekenhuis te Nieuwegein en na haar afstuderen begon ze daar als ANIOS. Tijdens deze periode werd de basis voor het uiteindelijke promotietraject gelegd. In september 2011won ze de Santeon wetenschapsprijs voor het onderzoek naar dexamethason bij een pneumonie. Vanaf 1 maart 2008 is ze gestart met haar opleiding tot internist in respectivelijk het St. Antonius ziekenhuis te Nieuwegein (opleider: Dr. A.B.M. Geers) en het Universitair Medisch Centrum Utrecht (opleider: Prof.dr. M.M.E. Schneider). Sabine woont samen met haar jeugdliefde Pascal en zij zijn sinds 5 september 2011 de trotse ouders van Hugo.