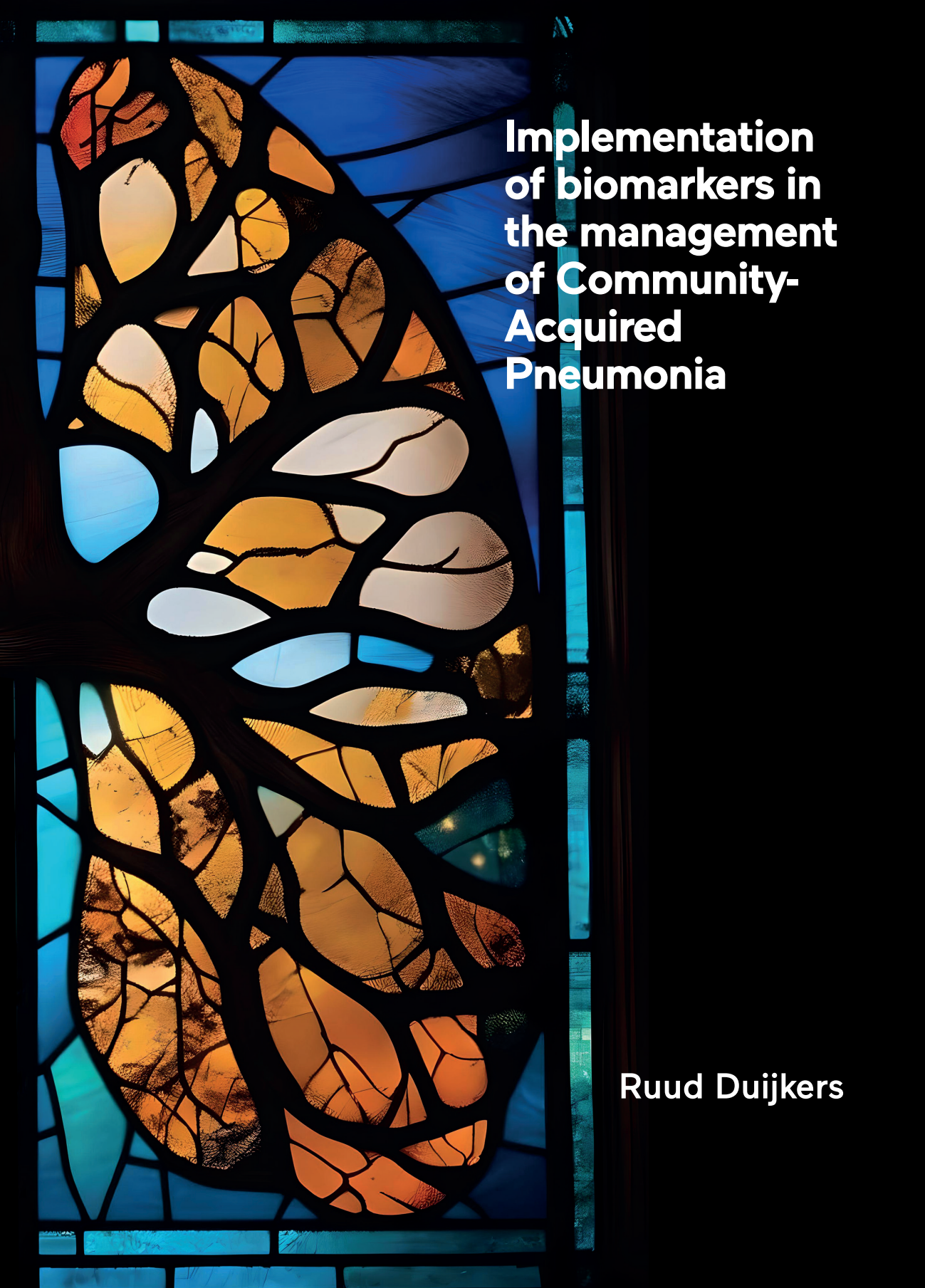

Implementation of biomarkers in the management of Community-Acquired Pneumonia

Ruud Duijkers

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"Implementatie van biomarkers in de behandeling van longontsteking opgelopen in de gemeenschap"

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Vormgeving en opmaak: Erik Elferink / Meneer E. illustratie en vormgeving
Cover: Ruud Duijkers
Organisatie: margreet@morganiseren.nl
Printing: Digiforce, Vianen

Printing of this thesis was supported by :
Noordwest Academie part of Noordwest Ziekenhuisgroep
Julius Center for Health Sciences and Primary Care

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"Implementatie van biomarkers in de behandeling van longontsteking opgelopen in de gemeenschap"

(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. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op donderdag 12 september 2024 des middags te 12.15 uur

door

Ruud Duijkers

geboren op 1 september 1987
te Zaandam

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Voor mijn vader

Chapter 1

General introduction

GENERAL INTRODUCTION

Background

Community acquired pneumonia (CAP) is defined as an acute infection of the pulmonary parenchyma acquired outside of a hospital setting. CAP is one of the leading causes of morbidity and mortality world-wide[1-3]. In Europe approximately 3.3 million people develop CAP each year, of whom 20-50% need hospital admission[4]. In the United States CAP accounts for over 4.5 million outpatient and emergency room visits annually of which approximately 1.5 million are admitted each year[5].
The annual costs associated with CAP are substantial. In Europe the annual costs are estimated around €10.1 billion, with inpatient care accounting for €5.7 billion and treatment accounting for €0.2 billion[4].
The clinical presentation of CAP varies, ranging from nearly asymptomatic to severe respiratory distress and sepsis. Because of the wide spectrum of associated clinical features, CAP is part of the differential diagnosis of nearly all respiratory illnesses. Diagnosis is reliant on demonstration of an infiltrate on chest imaging with a compatible clinical syndrome. However, this combination of findings is nonspecific and shared among many cardiopulmonary disorders[6,7].
Treatment for CAP consists of empiric antibiotic treatment, with guidelines recommending antibiotic courses of 5-21 days, depending on severity of illness, clinical response to treatment, causative pathogen (if identified) and type of antibiotic used[6-8].
In this thesis several diagnostic challenges in CAP are discussed, followed by the potential role biomarkers can play in management of CAP.

Pathogenesis and microbial aetiology of CAP

Historically, it was assumed that the lungs were sterile and CAP was caused by inhalation or microaspiration of bacterial or viral pathogens. Transmission was either via droplets or, in case of certain pathogens, aerosols. After eventually reaching the lower respiratory tract and pulmonary parenchyma, overwhelming of host defenses eventually lead to infection. The corresponding immune response, replication of pathogens and production of virulence factors lead to further inflammation and damage of the lung parenchyma[9].
Over the last few years the discovery of the lung microbiome has somewhat shifted this theorem, where resident microbes may compete with infecting pathogens or modulate immune-responses to other pathogens. Theoretically other insults to the lung microbiome such as smoke inhalation could cause a shift in the lung microbiome leading to dysbiosis and overgrowth of certain pathogens and eventually infection[9,10] .
Establishing a definite microbial diagnosis in patients with CAP is challenging. In up to 62% of hospitalized patients no pathogen is detected despite (extensive) microbiological evaluation[11-14]. Proposed explanations are increased use of antibiotics prior to diagnostic testing, or indeed overgrowth of the lung microbiome which is comprised primarily by anaerobic bacteria and microaerophilic streptococci which cannot be cultivated using standard culture methods[10,15-17].
Recommendations in guidelines for standard microbiological work-up of admitted pneumonia patients are largely based on expert opinion and differ worldwide[6-8].
Furthermore our knowledge of pathogens that cause CAP and distribution of pathogens worldwide are evolving, with some key factors being the introduction of pneumococcal vaccines, the COVID-19 pandemic and increased use of PCR techniques which are more sensitive in identifying atypical pathogens than conventional culture methods and have led to increased recognition of other respiratory viruses[11,18-23].

Challenges in diagnosis of S. *pneumoniae*

S. pneumoniae is the most common cause of CAP and estimated to cause 15-40% of CAP episodes[11,14]. The current diagnostic standard is comprised of blood cultures, sputum culture and urinary antigen tests (UAT). Antibiotic pretreatment greatly diminishes the yield from conventional blood and sputum cultures making them less reliable. In a clinical setting diagnosis of pneumococcal pneumonia based on sputum cultures alone is controversial due to the capability of S. pneumoniae to colonize of the upper respiratory tract[24,25].
The most widely used urinary antigen tests detect the C-polysaccharide antigen which is prevalent in all serotypes of S. pneumoniae. UAT is currently the test with highest sensitivity and specificity, ranging from 74-75% and 94-97% respectively, increasing the detection rate of S. pneumoniae in patients with CAP from 11.0 to 27.0%[24-28]. However the urinary antigen test has some limitations, false negative results can occur in relation with low levels of the C-polysaccharide antigen, false positive results can be induced by cross reaction with S. *viridans* species, asymptomatic colonization with S. pneumonia, recent vaccination for pneumococci and previous pneumococcal infection, where detectable levels of antigen were present in 40-50% of patients 1 month after infection[25,29,30].
Real-time quantitative PCR (rt-qPCR) has the potential to negate some of these problems, since it isn't affected by antibiotic pre-treatment and with a proper cut-off value should theoretically be able to distinguish between colonization and infection. For pneumococci several target genes are available, most commonly the pneumolysin gene (ply), autolysin (*lytA*), wzg (*cpsA)*, pneumococcal surface adhesion A (*psaA)* and Spn9802 gene fragment have been used as targets to detect S. pneumoniae[31]. A potential target gene should ideally be a stable or conserved gene, making it a favorable target for detection. Furthermore it should be specific for S. pneumoniae and ideally be absent in the other non-pneumococcal streptococci such as Streptococcus mitis, *Streptococcus oralis* and *Streptococcus pseudopneumoniae*[32].
Of the aforementioned genes *lytA* is a stable gene that encodes for an autolysin which is activated in the presence of antibiotics and certain detergents[33,34]. It is also considered to be a virulence factor, which enables S. pneumoniae to enter the cells of its host, replicate inside these cells and persist in them[35]. *LytA* is also present in S. mitis but gene sequences vary more among streptococcus species than among S. pneumoniae strains[36]. Interestingly, in a study where S. mitis was positive for *lytA* it was also associated with relevant respiratory disease which underlines its role as a virulence factor[37]. A drawback of *lytA* is that some strains, believed to be less than 2% of clinical isolates, of S. pneumoniae are bile-insoluble and produce negative results for *lytA* PCR due to alteration of the gene sequence[36].

In vitro and in vivo studies using *lytA* as a target for S. pneumoniae have shown promising results, with sensitivities and specificities ranging from 53-100% and 82-100% respectively[32,34,38-43].
One clinical study evaluating *lytA* rt-qPCR on nasopharyngeal samples using a cutoff of ≥8,000 copies/mL showed an increased proportion of CAP cases attributable to S. pneumoniae from 27.1 to 52.5% as compared to their reference standard of positive blood culture or sputum culture or UAT[38].
Despite these promising findings, there is still ongoing debate what the best sampling site should be. Studies have been done with whole blood samples, lower respiratory tract specimens, pleural fluid samples, oropharyngeal samples and nasopharyngeal samples. Of these, the oro- and nasopharyngeal samples are clinically probably the most interesting options, since they can be obtained in any patient and do not rely on expectoration of sputum or other invasive methods. Furthermore, an optimal cut-off value distinguishing colonization from infection has not been established and will likely differ between sampling sites and age groups.

Challenges in diagnosis of *Legionella*

Legionella infection is an important cause of CAP and estimated to account for approximately 1-10% of cases worldwide, with large geographical differences[11,13,44-47]. In both the EU and the United States, incidence of Legionella associated CAP is on the rise, with incidence quadrupling in the US[48,49]. The cause of this increase is not completely understood, but is likely in part due to better diagnostic methods, increased surveillance and identification of other pathogenic Legionella strains such as *L. Longbeachae* which is predominantly found in Australia, New Zealand and Asia, but has been detected in both the US and Europe[48-53].
CAP caused by Legionellae is most often diagnosed in hospitalized patients and can be severe. Up to 44% of patients have been reported to require ICU admission and associated reported mortality rates vary from 1-10%[54-56]. Data from observational studies suggest that delays in appropriate antibiotic therapy are associated with increased mortality[57-60]. Based on these data current consensus is that antibiotic coverage for Legionella should be initiated if there is a high clinical suspicion of Legionnaires' disease, if a diagnosis of Legionella is established or in patients with (moderate-)severe pneumonia[6-8].
However, diagnosis of Legionella-related CAP is difficult, because culturing Legionella from sputum and blood takes 3 to 10 days and has a low yield. The introduction of the urinary antigen test (UAT) for Legionella pneumophila improved diagnosis, especially in severe cases. However, the UAT can be negative in the early phase of the disease, especially in patients with mild disease. UAT only detects Legionella pneumophila serogroup 1 antigens, accounting for more than 80% of Legionella cases[48,49,61]. PCR in respiratory samples can improve diagnostic yield, but is reliant on expectoration of sputum or invasive diagnostic methods, which can be problematic since dry cough is considered frequently present in patients with Legionella-related CAP.
Several different clinical scoring systems have been developed over the years, aiming to aid clinicians in predicting or suspecting Legionella, but most have limited clinical significance because of low accuracy or the need to include follow-up date over several days[62-64]. Consequently, empiric antibiotic coverage based on these scoring systems may frequently be inadequate[6-8].Fiumefreddo et al. developed a diagnostic scoring system consisting of 6 items which are easily obtainable on admission, namely fever, dry cough, hyponatremia, elevated lactate dehydrogenase (LDH) and elevated C-reactive protein (CRP)[65]. In the derivation cohort, the diagnostic accuracy of the score was high, with an area under the curve (AUC)
of 0.86 (95% confidence interval (CI) 0.81–0.90). Validation studies found sensitivities and specificities ranging from 94-97% and 23-49% respectively[66-68]. Overall the total number of patients in these validation studies with Legionella-related CAP is relatively low (229 out of 3114) despite selection of patients. To date no prospective clinical impact studies using the score in practice have been performed.
In theory, if further validated, this prediction score could be a useful clinical tool to tailor empiric antibiotic treatment especially in cases where a (fast) microbial diagnosis is not obtained or challenging.

Challenges in diagnosing viral CAP

Introduction of PCR has increased our ability to detect respiratory viruses. To date at least 26 viruses have been associated with community-acquired pneumonia[69]. Even prior to the COVID-19 pandemic a viral pathogen could be identified in 20-50% of patients with CAP[11,69-71].
Unfortunately a standardized definition of "viral pneumonia" is lacking, making literature hard to interpret. Especially studies relying solely on identification of a viral pathogen in absence of a bacterial pathogen are at risk of misclassifying cases as viral pneumonia due to the low yield of other conventional microbiological tests. Viral pathogens can predispose patients for concomitant bacterial infection via specific immune pathways which will be discussed below, further increasing the risk of misclassification of cases.
If respiratory viral pathogens are detected by PCR, these viral agents can be causative or non-causative for infection. When causative, they can be in fact coinfections with undetected bacterial pathogens or strict viral infection. 'Strict viral' CAP is defined as when a respiratory virus is the only causative pathogen for CAP in a patient. For strict viral CAP, antibiotics are probably ineffective and in theory should be withheld. Unfortunately radiological features of viral pneumonia are non-specific and can't discriminate viral from bacterial CAP[72,73]. Symptom-based prediction of aetiology has also proven inadequate to discriminate between viral and bacterial aetiology[74-77].
In the early phase of pneumonia, bacteria and viruses trigger distinct innate immune response pathways. In bacterial CAP, rapid interleukin-17A (IL-17A) production by gamma-delta T cells attracts, expands and activates neutrophils at the site of infection[78]. Release of young neutrophils from the bone marrow is an innate response aimed at mainly extracellular pathogens, such as pneumococci[79]. IL-6 enhances general pro-inflammatory activity as well as T-helper-17 (Th-17) development from naïve T cells[80]. In bacterial pneumonia higher levels of IL-6, tumour necrosis factor α (TNF-α) and interleukin-1 (IL-1) were found in bronchoalveolar lavage (BAL) compared to healthy controls. Also in serum, IL-6 was elevated in bacterial CAP compared to healthy individuals[81].
In viral CAP, type 1 interferons (IFN) are produced by infected cells, often airway epi-

thelial cells. Natural Killer (NK) cells and also CD8 T cells will produce type 2 interferon (IFN-γ) in response to viral replication. Interferons limit viral replication and enhance the T-helper-1 pathway response. However, T-helper-2 pathway cytokines, such as IL-5, results in the recruitment and activation of eosinophils, which can display anti-viral activities as well[82-84].

In mixed bacterial/viral infections, primary viral pathogens can enhance bacterial infection. Adherence of bacteria to epithelial cells is enhanced in virus-infected cells, predisposing to superinfection, but several alternative mechanisms have been proposed[85]. CAP patients with primary influenza infection have elevated levels of IL-27, which in turn inhibit IL-10 production and therewith the Th-17 pathway. This does not alter viral clearance, but limits lung neutrophil influx and potentially diminishes bacterial clearance[86-88]. Due to these pathways procalcitonin (PCT) has been extensively studied over the years for its potential to discriminate between viral and bacterial infections. Procalcitonin is released in multiple tissues in response to several cytokines such as IL-1β, TNF-α and IL-6. Conversely PCT production is blocked by IFN-γ[89]. This means PCT is markedly elevated in response to bacterial infections and production should remain relatively low in viral infections.

Unfortunately, PCT levels are relatively low in atypical bacterial pathogens and can be markedly elevated in severe respiratory viral infection without presence of a bacterial pathogen[90-93].This makes procalcitonin unfit as a biomarker to predict aetiology in individual patients, with a meta-analysis of 12 studies resulting in an AUC of 0.73[90,94].

Due to the distinct differences in immunological pathways, a combination of cytokines/biomarkers on admission perhaps might be able to differentiate between different aetiologies of CAP.

Biomarkers and treatment of CAP

Current CAP guidelines suggest antibiotic courses of 5-21 days depending on severity of illness, causative pathogen, clinical response and type of antibiotic used[6-8]. Studies have shown that antibiotic treatment duration can be shortened based on clinical parameters, such as the criteria for clinical stability defined in the IDSA guidelines, or based on clinical improvement to initial antibiotic treatment[6,95-97].

Yet, in daily practice physicians tend to treat longer than recommended, especially in patients with significant comorbidities, in patients who fail to respond rapidly on antibiotic treatment and in patients with severe CAP[95,98-100]. Several studies and reviews in hospitalized CAP patients consistently show a median treatment duration between 7-10 days, meaning often patients receive longer antibiotic courses[95-97,101-106].

Antibiotic (over)use is an important driver of emergence of antimicrobial resistance, which is considered one of the most urgent threats to global health[107]. This underlines the need for guidance to shorten the duration of antibiotic treatment without compromising patient safety.

Over the years efforts have been made to reduce antibiotic overuse by means of antibiotic stewardship programs which focus on adherence to guidelines. However, these programs are often difficult, expensive and time-consuming to implement and effects often diminish when the programme ends[108,109].

Biomarkers have been proposed as objective means to tailor antibiotic treatment in

patients with CAP. PCT is among the most studied biomarkers in CAP and C-reactive protein (CRP) is probably used most in day-to-day clinical practice.

Procalcitonin is normally only synthesized in the thyroid gland where it serves as a precursor for the calcitonin protein. In absence of systemic inflammation procalcitonin is not released into the blood, resulting in low serum levels in healthy individuals[110,111].

In response to an inflammatory stimulus PCT is widely synthesized outside of the thyroid gland and blood levels increase after about 2-4 hours, typically peaking after 24-48 hours. PCT has a half-life of about 25-30 hours, meaning a decline of about 50 percent each 1 to 1.5 days when inflammation resolves[112,113]. Procalcitonin is usually markedly elevated in typical bacterial infections due to immunological pathways that have been described above. Atypical pathogens often cause a lesser increase in PCT than typical bacterial pathogens[90,114,115].

However several other infectious and non-infectious causes of systemic inflammation have been known to induce procalcitonin production. Among other infectious causes are severe viral infections, candida species, pneumocystis jirovecii and parasites such as malaria[92,116-122]. Non-infectious causes include circulatory failure, burns, trauma, surgery, pancreatitis, intracranial hemmorhage, certain neoplasms, severe liver disease, renal impairment, some auto-immune disorders and certain immunomodulatory agents[123-135].

Patients with kidney disease have higher baseline circulating levels of procalcitonin, which is believed to be due to higher levels of circulating inflammatory cytokines. The elimination of procalcitonin in patients with renal failure is prolonged compared to healthy individuals[136,137]. PCT is the most studied biomarker when it comes to tailoring antibiotic treatment in patients with CAP and seems useful to withhold or discontinue antibiotics in patients with acute respiratory infections, including CAP, without an increase in treatment failure or mortality[138,139].

However, concerns have been raised regarding patient selection in clinical trials, adherence to PCT algorithms by treating physicians varying between 44-84% and usefulness of PCT in patients with atypical pathogens or renal failure[90,140,141].

C-reactive protein is an acute phase protein that is synthesized by hepatocytes under the influence of other pro-inflammatory cytokines, mainly IL-6, IL-1β and TNF-α in response to infections, inflammation and tissue injury[142]. CRP has both pro-inflammatory and anti-inflammatory actions and promotes the recognition and elimination of pathogens and enhances clearance of necrotic and apoptotic cells[142,143].

CRP is commonly used in day-to-day management of patients with CAP, however much less is known about its potential role in tailoring antibiotic treatment. Results from observational studies in patients with CAP suggested that CRP might aid the clinical decision-making process[144,145]. Failure to decline of CRP in the first 3 days of hospitalization is associated with poor outcomes[146].

CRP has been used successfully in a variety of other settings, mostly in patients with lower respiratory tract infections in primary care or a nursing home setting. However, the focus has primarily been on initiating or withholding antimicrobial treatment and not tailoring of antibiotic treatment duration[147,148].

Only once has a CRP-based treatment duration algorithm been compared to a PCT-based algorithm[149]. This study included 94 ICU patients patients with sepsis, 49 were allocated to PCT and 45 to CRP measurements, without a control group. Median duration of treatment was 6 days in the CRP group and 7 days in the PCT group, with no diffe-

rences in outcomes between groups. The same group studied a modified version of their CRP algorithm and compared it to best-practice care in an open label RCT in ICU patients and found a small reduction in antibiotic treatment time in favour of the CRP group[150].
Overall the role of biomarkers in determining optimal treatment duration for hospitalized patients has not been established and important questions remain.

Risk-stratification in CAP and biomarkers

Risk stratification for patients presenting to emergency departments with CAP is important to determine the optimal care strategy, including decisions for admission to hospital wards, ICU or ambulatory care. Clinicians frequently use risk scores such as the CURB-65 and PSI score, which have been developed for predicting CAP-associated short-term mortality[151,152]. Another approach is more pragmatic and defines severity as mild, moderate or severe based on either ambulatory treatment, treatment in a regular hospital ward, or ICU ward respectively. Concordance between these classification systems is poor, with patients classified as severe pneumonia in 22% of cases using CURB-65 score, 13% with the PSI score and only 3% using the pragmatic classification[153]. Both risk scores have their limitations. The PSI-score is largely age-driven and tends to overestimate severity in elderly patients and the CURB-65 score has a relatively low predictive value for patients needing critical care interventions[154,155].
Multiple biomarkers have been tested for their capacity to improve prognosis prediction in patients with CAP. Both CRP and PCT appeared useful to predict typical bacterial pathogens, assessing severity, predicting mortality risk and potential complications of CAP in some studies[90,139,156].
One of the less frequently tested biomarkers is midregional proadrenomedullin (MR-proADM).
In the 1980s MR-proADM was identified in high concentrations in septic patients[157]. MR-proADM is a protein derived from the same precursor as adrenomedullin. Adrenomedullin is a more physiologically active protein than MR-proADM with important vasodilatory, immune modulatory and anti-microbial effects[157]. However, adrenomedullin itself cannot be reliably measured in plasma due to a short half-life, immediate binding to receptors in the vicinity of its production and existence of a binding protein in plasma[158,159].
In several studies MR-proADM appeared as a useful tool for risk stratification in CAP patients, enhancing accuracy of existing risk scores, independent of aetiology and able to predict complications and possibly long-term outcomes[160-162].
Yet in a randomized trial among patients presenting to the emergency department with lower respiratory tract infections a strategy to triage and discharge patients based on medical and biopsychosocial risk assessment in conjunction with MR-proADM levels failed to reduce length of stay and adverse outcomes, compared to a strategy not using MR-proADM. However, the study algorithm was overruled in 39.3% of patients at presentation and in 34.5% during hospitalization[163].
Whether use of MR-proADM improves patient outcomes is yet to be determined and an optimal cut-off value for MR-proADM for decision-making is not known. Most studies using MR-proADM have focused on predicting mortality or complications from CAP, such as requiring ICU-admission. This might diminish the prognostic usefulness of MR-proADM for patients admitted to regular hospital wards with regards to other relevant outcomes.

THESIS AIM AND OUTLINE

The principal objective of this thesis was to optimize the use of antibiotics in the management of patients with CAP admitted to a regular hospital ward by means of biomarker guided treatment algorithms. We compared a new CRP algorithm to an extensively validated PCT algorithm and routine clinical care. During a 30-day follow-up period, we looked at the influence of both algorithms on total duration of antibiotic treatment, new antibiotic prescriptions, length of stay, time to clinical stability and all-cause mortality. This study is presented in **chapter 5**. The design of this study makes it applicable to a great number of patients in clinical practice and has several features that mimic the real-life situation. First, our selection of patients included all patients with a diagnosis of CAP made by the attending physician rather than after consultation of a radiologist or senior physician, which reflects routine clinical practice. Second, our exclusion criteria were limited to patients who would not be treated as regular CAP patients according to the Dutch guidelines. Third, we did not exclude patients who were pre-treated with antibiotics. Lastly, biomarker samples were always taken during regular morning rounds irrespective of time of admission and antibiotic duration was pragmatically scored in days instead of dosages which makes it easier to implement and translate to a real-life clinical setting.

Chapter 2 describes the development and validation of a real-time quantitative PCR for S. pneumoniae and describe its performance in a pilot study using a select subset of patients with pneumococcal pneumonia and other pathogens.

Chapter 3 focuses on validation of an existing legionella prediction score which might be a useful clinical tool for clinicians to suspect Legionella pneumonia and guide empiric antibiotic treatment, especially in cases that are not detected by urine-antigen tests. Our study uses the largest cohort of legionella patients to date and describes its performance in a cohort of CAP patients.

In Chapter 4 we investigated whether plasma cytokine levels of the Th17, Th1 and Th2 pathways can be used to distinguish between three aetiological groups: pneumococcal, viral and mixed viral/bacterial infection. First, we investigated absolute cytokine plasma-level differences between groups. Hereafter, we investigated whether cytokine-based prediction models can be used to differentiate viral CAP from other aetiologies at admission, and whether this adds value to the routine determination of CRP.

Lastly in **chapter 6** we aimed to determine differences in MR-proADM levels on admission between matched cases and controls, focusing on short-term adverse outcomes, including treatment failure, short-term mortality, and re-admission after discharge in patients admitted with CAP to a non-ICU hospital ward.

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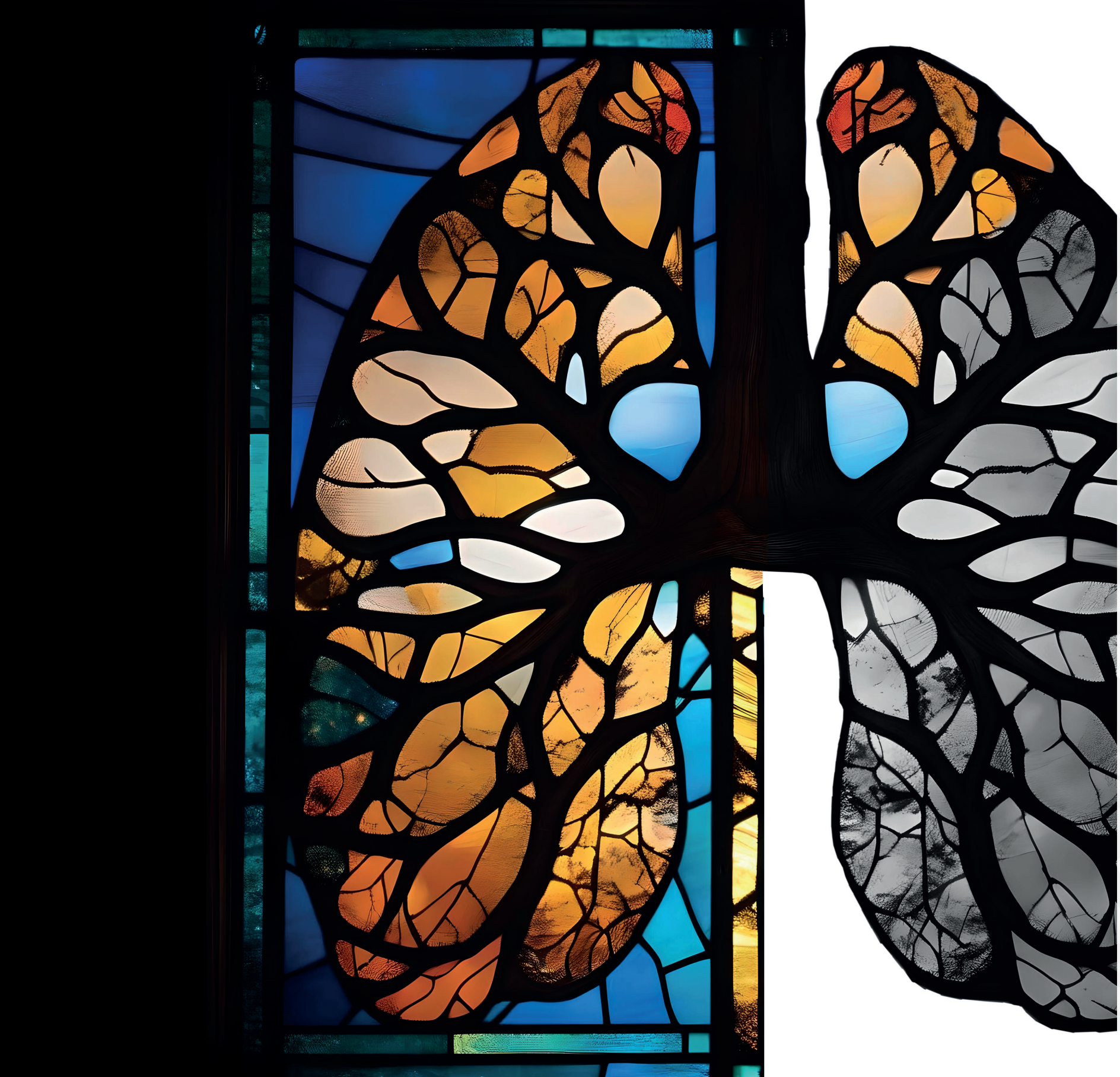

Chapter 2

Feasibility of a quantitative polymerase chain reaction assay for diagnosing pneumococcal pneumonia using oropharyngeal swabs

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Molecular Biology Reports (2019) 46:1013–1021

ABSTRACT

Introduction

Streptococcus pneumoniae is the most important pathogen causing community-acquired pneumonia (CAP). The current diagnostic microbial standard detects *S. pneumoniae* in less than 30% of CAP cases. A quantitative polymerase chain reaction (PCR) targeting autolysin (*lytA*) is able to increase the rate of detection. The aim of this study is validation of this quantitative PCR in vitro using different available strains and in vivo using clinical samples (oropharyngeal swabs).

Methods

The PCR autolysin (*lytA*) was validated by testing the intra- and inter-run variability. Also, the in vitro specificity and sensitivity, including the lower limit of detection was determined. In addition, a pilot-study was performed using samples from patients (n=28) with pneumococcal pneumonia and patients (n=28) with a pneumonia without detection of *S. pneumoniae* with the current diagnostic microbial standard, but with detection of either a viral and or another bacterial pathogen to validate this test further.

Results

The intra- and inter-run variability were relatively low (SD's ranging from 0.08 to 0.96 cycle thresholds). The lower limit of detection turned out to be 1-10 DNA copies/reaction. In-vitro sensitivity and specificity of the tested specimens (8 strains carrying *lytA* and 6 strains negative for *lytA*) were both 100%. In patients with pneumococcal and non-pneumococcal pneumonia a cut-off value of 6.000 copies/ml would lead to a sensitivity of 57.1% and a specificity of 85.7%.

Conclusions

We were able to develop a quantitative PCR targeting *lytA* with good in-vitro test characteristics.

INTRODUCTION

Streptococcus pneumoniae is the most important pathogen causing community-acquired pneumonia (CAP)[1–3]. The current diagnostic standard, comprised of blood cultures, sputum cultures and the urinary antigen test (UAT), is only able to detect *S. pneumoniae* in less than 30% of CAP cases[4, 5]. Furthermore, it takes up to several days to yield a positive result and antibiotic therapy can be narrowed[6]. The UAT is currently the test with the highest sensitivity, ranging from 59 to 87% and specificity of 94%, increasing the detection of *S. pneumoniae* in patients with CAP from 14.0% to 27.0%[7]. Detecting *S. pneumoniae* before or after the start of antibiotic treatment requires a target. Different genes of *S. pneumoniae* have been used in research as a target, including spn9802, pneumolysin (ply), wzg (*cpsA*), and autolysin (*lytA*) by PCR[4,5, 8–18]. A target gene should be specific for *S. pneumoniae* and be absent in the other non-pneumococcal streptococci such as *Streptococcus mitis, Streptococcus oralis* and the recently discovered *Streptococcus pseudopneumoniae*[18]. Remarkably, ply is believed to be less specific for *S. pneumoniae* than *lytA*[19, 20]. The ply and *lytA* gene have both been found in *S. mitis* strains. The isolates containing these genes were all associated with respiratory disease[21]. One recent study by Albrich et al[5]. showed that quantitative polymerase chain reaction (qPCR) tested on nasopharyngeal (NP) samples targeting the *lytA* gene in a study population that consisted mainly of HIV-infected adults detected *S. pneumoniae* in 52.5% of CAP cases. The diagnostic standard (blood culture, sputum Gram stain or culture or UAT) detected *S. pneumoniae* in only 27.1% of CAP cases. The combination of target genes has been suggested to improve the reliability of the qPCR. The target gene piaB has been used next to *lytA* to increase the specificity. A recent study by Simoes et al.[22] used both *lytA* and *piaB* to identify *S. pneumoniae* and the addition of piaB led to the discovery of two pneumococcal strains that were not identified by *lytA* alone. However, the authors mention that piaB is not present in some non-encapsulated pneumococci and some non-typeable pneumococci. An earlier study also combined *lytA* and *piaA* for the detection of colonization of the nasopharynx by *S. pneumoniae*[23]. A strain was considered to be a *S. pneumoniae* species when both genes were present. Four strains did not include the *piaA* gene, but turned out to be *S. pneumoniae* species.

Using two target genes leads to the difficult situation of interpreting a strain which encompasses one gene, but lacks the other gene. Adding *piaB* will lead to a lower sensitivity. This means that some patients will be withheld narrow-spectrum antibiotics.

LytA encodes for an autolysin that is activated in the presence of antibiotics such as penicillin and detergents such as deoxycholate[24]. It has also been considered to be a virulence factor, which means that it enables *S. pneumoniae* to enter the cells of its host, replicate inside these cells and persist in them[25]. *LytA* is a stable or conserved gene, which is a favorable target for detection[13]. In 2001 McAvin et al.[13] found that in vitro the *lytA* gene showed promising results with a sensitivity and specificity of 100% for *S. pneumoniae*. A more recent study in which clinical samples were used also found a specificity of 100%, but a much lower sensitivity of 53%[26]. Other research stated that

lytA is not specific enough to differentiate between *S. pneumoniae* and some strains of S. mitis, *S. pseudopneumoniae* and *S. oralis*[18,27,28]. However, there are studies that claim that *lytA* can rarely be found in non-pneumococcal bacteria[14,15].

Streptococcus pneumoniae is a pathogen capable of colonization of the upper respiratory tract[23,29]. Differentiating between colonization and infection is necessary to detect the patients with true pneumococcal pneumonia. Setting a cut-off value using a qPCR could potentially deal with this problem.

The aim of our study is to set up and validate a quantitative PCR assay targeting the *lytA* gene for detection of *S. pneumoniae* in adult patients with CAP. First, we validated the assay by examining the quality and reproducibility. Subsequently, the sensitivity and the lower limit of detection of the assay, as well as the specificity of the PCR was tested. After validation, we performed a pilot-study with clinical samples in patients with pneumonia caused by different pathogens.

MATERIALS AND METHODS

Study outline

The study was performed in the Regional Laboratory for Public Health Kennemerland in Haarlem between the 1st of September and the 8th of December 2015.
Amplification of a part of the bacterial DNA (the amplicon) using PCR leads to extremely high levels of amplicons after the experiment, in contrast to relatively low levels before the start of the amplification cycles. To check for possible inaccuracy the qPCR assay was compared with one other method of quantification: quantification using universal 16S ribosomal RNA primers. The concentration of the sample that was used containing a quality control strain of *S. pneumoniae (S. pneumoniae* American Type Culture Collection (ATCC) 49619) was calculated with PicoGreen quantification and 16S rDNA quantification. This calculated concentration was used to asses a standard curve for a *lytA* qPCR using primers/probe constructed by Carvalho et al.[26]; forward primer (560nM): 5'-ACGCAATCTA GCAGATGAAG CA-3'; reverse primer (2800nM): 5'-TCGTGCGTTT TAA-TTCCAGC T-3'; probe (700nM): 5'-FAM- GCCGAAAACG CTTGATACAG GGAG-3'-BHQ1. The standard curve enabled calculation of concentrations from other samples of *S. pneumoniae* and other non-pneumococcal streptococci (provided by the Department of Paediatric Immunology and Infectious Diseases, Wilhelmina's Children Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands). The concentrations of the standard curve were compared to those calculated using 16S rDNA quantification performed by aforementioned samples.

Bacterial strains

Streptococcus pneumoniae ATCC 49619 was used to compare methods and for optimization of the quantitative PCR targeting *lytA* as well as assessing a standard curve. A collection of strains was used to test the specificity and sensitivityof the assay.
S. pneumoniae strains with known concentrations (OK-2-816; OK-2-1213; OK-2-1214; OK-2-077) and unknown concentrations (serotype 8; serotype 14; serotype 19A; strain 406) were used to test the sensitivity. *S. pseudopneumoniae* strains (k221; lLI42; OK-3-VE-224; 2120942), as well as a *S. mitis* (*S. mitis* SK579 (b1019)) and a *S. oralis* strain (2021933), all *lytA* negative, were used to test the specificity. The strains were provided by the Department of Paediatric Immunology and Infectious Diseases, Wilhelmina's Children Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands. Concentrations and characteristics are available from the supplementary appendix.

Clinical samples

Samples were prospectively collected from patients with CAP (REDUCE study; clintrials.gov database NCT01964495). For this present (pilot-)study oropharyngeal (OP) swabs were used. All oropharyngeal swabs were obtained by rolling the swabs on the tonsils and posterior wall of the oropharynx with enough pressure to dislodge cells from the mucosal surface. The oropharyngeal swabs used in this study are eSwab™ with liquid Amies medium as preservation medium (Copan Italia SpA, Brescia, Italy). Viral pathogens could be identified using a PCR performed on these OP swabs. Of every swab 5 µL liquid was added to the primer/probe mix. The patient characteristics are shown in Table 2.1

Table 2.1 Patient Characteristics

	Patient characteristics			
	S. pneumoniae (n=28)		Other pathogens (=28)	
Age (year)	67.38±16.218 (Range 24–92)		67.54±13.226 (Range 44–94)	
Male	16	57.1%	16	57.1%
Female	12	42.9%	12	42.9%
Current smoker	9	32.1%	6	21.4%
Previous smoker	13	46.4%	16	57.1%
CURB-65				
0	5	17.9%	8	28.6%
1	5	17.9%	11	39.3%
2	10	35.7%	6	21.4%
3	8	28.6%	2	7.1%
4	0	–	1	3.6%
5	0	–	0	–
COPD	11	39.3%	14	50%
Pre-treatment with AB	2	7.1%	7	25%
Positive blood culture	12	42.9%	3	10.7%
Positive sputum culture	10	35.7%	11	39.3%
Positive urinary antigen test	13	46.4%	1[a]	3.6%
Positive pharyngeal swab (viral pathogens)			20	71.4%

The patient characteristics of the two groups (infected with *S. pneumoniae* or other viral/bacterial pathogens) of patients admitted with CAP. AB antibiotics. Pharyngeal swabs were only tested for viral pathogens at time of admittance
[a] Positive for Legionella pneumophila

Isolation of bacterial DNA

DNA of *S. pneumoniae* ATCC 49619 was isolated using the Highpure PCR template preparation kit (Roche Diagnostics Nederland BV, Almere, The Netherlands). Bacterial DNA from clinical respiratory samples were obtained by total DNA extraction using a NucliSENS EasyMag total nucleic acid extractor (bioMérieux, Marcy l'Etoile, France). The total nucleic acid component of the sample (200 µL) was eluted in a final volume of 40 µL.

Molecular quantification of bacterial DNA of the positive control *(S. pneumoniae ATCC 49619)*

For quantification of a positive control (*S. pneumoniae* ATCC 49619) we used the Quant-IT PicoGreen dsDNA assay kit (Life Technologies, Bleiswijk, The Netherlands). The fluorescence was measured using a LightCycler®480II real-time PCR analyser (Roche, Almere, The Netherlands). To convert from the concentration in ng/µL to the number of genome copies per µL the genome size, approximately 2.1 million base pairs was estimated[30].

As a comparison for the Picogreen quantification method, quantification of *S. pneumoniae* ATCC 49619 was performed using a 16S PCR targeted by universal primers[31]. The quantitative PCRs were run on LightCycler 1.5 or 2.0 (Roche, Almere, the Netherlands). LightCycler software (Version 4.1) resulted in the calculation of the number of *S. pneumoniae* DNA copies of the positive control.

Quantitative PCR targeting *lytA*

The *S. pneumoniae*-quantitative PCR uses primers targeting the *lytA* gene as described by Carvalho et al[26]. Roche LightCycler® 480 Probes Master mix was used for all PCR reactions. PCR ran the following program using the LightCycler® 480 (Roche): 10 min at 95 °C, followed by 45 cycles that are comprised of 15 s at 95 °C and 1 min at 60 °C. A standard curve was assessed for the quantitative assay by using the *S. pneumoniae* ATCC 49619 strain. Standard curves (three standard curves, calculated with three different experiments, the average of these curves was used as a final standard curve) were validated using strains with known concentrations (OK-2-816; OK-2-1213; OK-2-1214; OK-2-077), kindly provided by the Department of Paediatric Immunology and Infectious Diseases, Wilhelmina's Children Hospital, University Medical Centre Utrecht, Utrecht, the Netherlands. Inter and intra-run variability were established by determination of triplicate serial dilutions in three independent runs.

Sensitivity of the *lytA* PCR

LLOD of the quantitative PCR was determined by multiple serial dilutions of purified DNA from *S. pneumoniae* ATCC 49619 equivalent to from 17,000 to 0.17 copies per µL.

Specificity of the *lytA* PCR

Specificity of the *lytA* real-time PCR was defined by testing purified DNA from eight pneumococcal strains. These strains include 4 strains that were non-typeable by culture (OK-2-816; OK-2-1213; OK-2-1214; OK-2-077) as well as serotype 8, serotype 14, serotype 19A and strain 406.

Further determination of the specificity was performed by using 6 strains including 4 strains of *S. pseudopneumoniae* (k221; ILI42; OK3-VE-224; 2120942), 1 strain of *S. mitis* and 1 strain of *S. oralis*. None of these strains encloses the *lytA* gene.

Statistical analysis

The 2 different methods (the concentrations calculated with the *lytA* qPCR and 16S rDNA quantification) were compared using the Bland–Altman-method[32]. Inter-run variability was calculated by one-way analysis of variance (ANOVA)[33,34]. Intra-run variability was also tested by one-way analysis of variance. SPSS statistical software (SPSS version 23 for Windows, Chicago, IL, USA) was used to perform the statistical tests mentioned above. A p-value < 0.05 was considered as statistically significant.

RESULTS

Molecular quantification of the positive control (*S.pneumoniae* ATCC 49619)

The first step in the validation was molecular quantificationof the positive control (S. pneumoniae ATCC 49619). Concentrations calculated with PicoGreen had and 16S rDNA experiments resulted in an average concentration of 1.70×10^6 DNA copies/µL.

Validation of the *lytA* quantitative PCR

The calculated average concentration of *S. pneumoniae* (ATCC 49619) was used to assess a standard curve. This standard curve had a slope of approximately - 3.4 and an efficiency of 95.1%. Four *lytA* positive strains with known concentrations were used to validate our standard curve.

Intra- and inter-run variability

To examine the feasibility of the qPCR as a diagnostic tool for CAP, the specificity and sensitivity characteristics are determined. Serial dilutions of *S. pneumoniae* (ATCC 49619) were used to account for intra- and interrun variability. For each step dilution, the standard deviation was calculated. Standard deviations ranged from 0.08 Ct-value for the samples with the highest concentration to an average of 0.96 Ct-values for the lowest concentration (0.17 DNA copies/µL or 170 DNA copies/mL). No significant differenceswere found when testing the inter-run variability with a one-way analysis of variance (ANOVA; p-value ranging from 0.426 to 0.929).

In vitro performance of the *lytA* quantitative PCR

In total 6 *lytA*-negative strains were tested. Four strains showed fluorescence, while 2 other strains showed no fluorescence after 45 cycles (supplementary appendix). The 4 *lytA*-negative strains that did show fluorescence appeared as multiple groups of DNA fragments (shorter than the amplicon of 75 base pairs), meaning they contained an accumulation of waste products. Dilutions of *S. pneumoniae* (ATCC49619) were used to establish the lower limits of detection (LLOD) of the qPCR targeting *lytA*. The LLOD ranged from approximately 0.85 (SD 0.96 Ct) DNA copy to approximately 8.5 (SD 0.36 Ct) DNA copies per well.

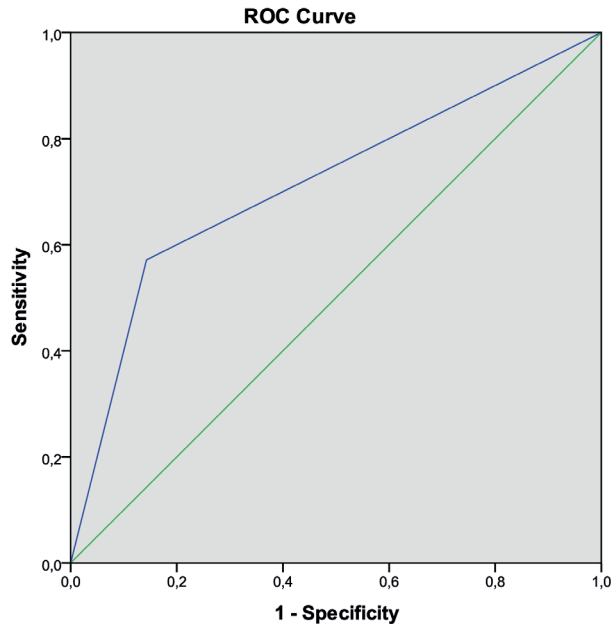
In addition, an attempt was made to identify 8 different *lytA*-positive *S. pneumoniae* strains. These different strains were all identified with Ct values ranging from 18 to 25 cycles. Only one strain had a positive result after approximately 36 amplification cycles (Serotype 19A). Testing the *lytA*-positive and *lytA*-negative strains resulted in an in-vitro sensitivity and specificity of both 100%. The LLOD was 1-10 copies/reaction.

Pilot-study of in vivo specimens

OP samples from 28 patients with CAP caused by *S. pneumoniae* and 28 patients with a viral pneumonia or pneumonia with another bacterial pathogen, identified by a positive blood, sputum culture or UAT result were used for this pilot study. Concentrations in the OP swabs tested in the group with *S. pneumoniae* ranged from 0 to 1190 DNA copies/µL; 5 patients had a negative result. Concentrations in the group with other pathogens ranged from 0 to 210 DNA copies/µL; 18 patients had a negative result. The largest Area Under the Curve (AUC) was found for a cut-off value of 6.000 DNA copies/mL (AUC 0.714 with a sensitivity of 57.1% and a specificity of 85.7%) (Figs. 2.1, 2.2) with a positive predictive value of 80% and a negative predictive value of 66.7%.

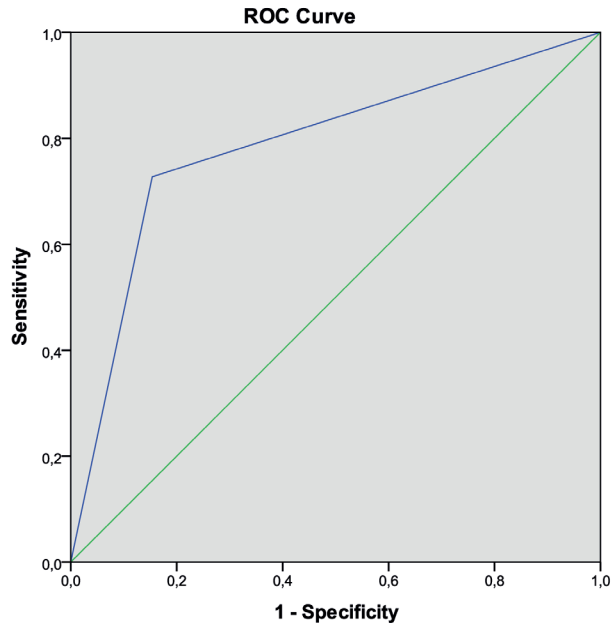
Eleven patients in the *S. pneumoniae* group and 13 patients in the group with other pathogens had a complete composite diagnostic microbial standard (blood culture, sputum culture and UAT). The range of the *S. pneumoniae* group (11 patients) was 0–145 DNA copies/µL with one negative result and the concentrations in the group with other pathogens (13 patients) ranged from 0 to 211 DNA copies/ µL, with eight negative results. The AUC for this second comparison was also highest with a cut-off value of 6.000 DNA copies/mL (AUC 0.787, with a sensitivity of 72.7% and a specificity of 84.6%). The positive and negative predictivevalue were 80% and 78.6% respectively.

Figure 2.1a



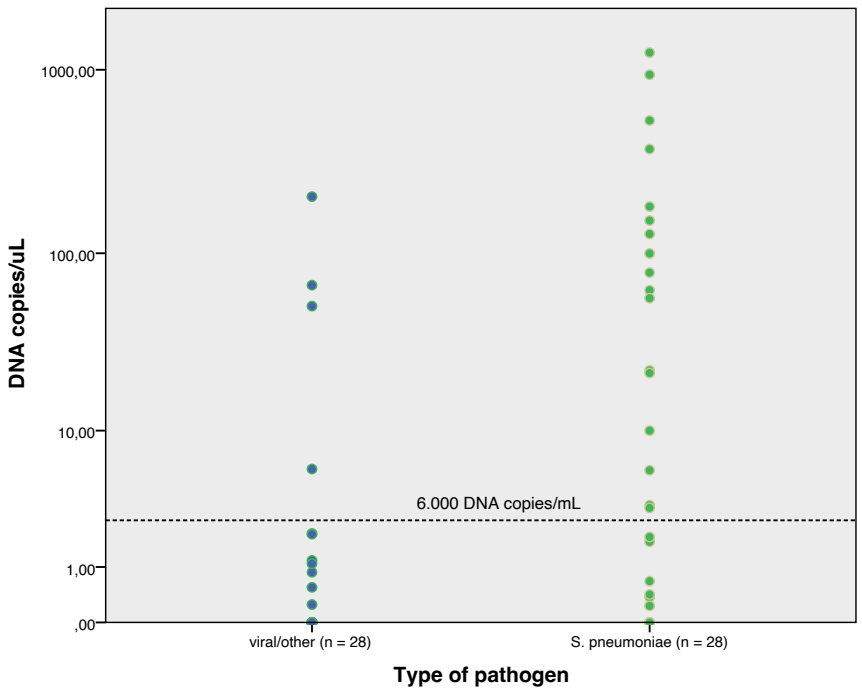
ROC-curve with cut-off value 6.000 copies/mL. Sensitivity is 57.1% and specificity is 85.7%. AUC is 0.714.

Figure 2.1b



ROC-curve with a cut-off value of 6.000 copies/mL. Only samples from patients with a complete composite diagnostic standard (blood culture, sputum culture and urinary antigen tested) performed were used for this curve. Sensitivity is 72.7% and specificity is 84.6%. AUC is 0.787

Figure 2.2



Pneumococcal load in oropharyngeal swabs. Number of DNA copies/microliter in oropharyngeal swabs in patients with confirmed pneumococcal pneumonia (n = 28) or viral/other pathogens (n = 28). The dotted line represents the cut-off value of 6.0000 DNA copies/mL

DISCUSSION

The present study shows that the *lytA* quantitative PCR is areliable test in order to detect *S. pneumoniae* in vitro and has the potential to be a reliable test in vivo. In vitro sensitivity and specificity are both 100%. More important the test shows promising results in differentiating between infectionand colonization. When tested on a small sample of patients, with a complete diagnostic work-up, a sensitivity and specificity of 72.7 and 84.6% respectively were reached using acutoff value of 6.000 copies/mL. One would expect a low number of DNA copies in patients with colonization without infection. With a sensitive test, which can detect a low number of DNA copies per microliter and makes it possible to set a low cut-off value when this hypothesis is true. The in-vitro LLOD turned out to be between approximately 1 and 10 copies/µL, which is similar to the LLOD's found by others varying from < 10 copies per reaction to 4.3 copies per reaction[12,15,26]. This LLOD makes the differentiation between colonization and infection possible. The standard deviations of our standard curve illustrate the reproducibility of our test.

The specificity and sensitivity are based on a total number of just 14 strains, which is a drawback of our study. In other studies a much larger numbers of pneumococcal strains and controls were tested[13–15]. An in-vitro specificity and sensitivity of 100% in the first study was found using 70 positive controls and 9 non-pneumococcal streptococci (including 2 *S. mitis* strains)[13]. This 100% specificity was confirmed by another study using 23 non-pneumococcal streptococci (including three that closely resemble *S. pneumoniae*; 2 *S.oralis* strains and 1 *S. mitis* strain)[14]. The largest study tested a total of 257 strains belonging to 37 different species including 30 *S. mitis* strains, with no false negative results and only one false positive result out of 30 *S. mitis* strains. This sample was also tested positive by two rapid antigen tests (Wellcogen and Phadebact)[15].

A recent study using the same positive control (ATCC 49619), primers and probe, tested 23 *S. pneumoniae* strains and 29 negative controls (including six non-pneumococcal species, one being *S. mitis*)[35]. The six negative controls used in the present study are all six closely related to *S.pneumoniae*. Testing these non-pneumococcal strains makes for a valuable contribution to previous trials because they generate signals reported specific to *S. pneumoniae*, in terms of optochin susceptibility, bile solubility, and Quellung testing, the classic methods used to identify pneumococci. These signals make it difficult to discriminate them from pneumococcal strains when performing these tests on blood cultures. However, our PCR was able to discriminate between these strains and *S. pneumoniae*. The small number of strains tested might overestimate the true specificity. The specificity could be improved by adding a *piaB* confirmation-PCR, which can be used for the samples tested positive for *lytA*.

Our pilot-study consisted of a only small number of patients admitted with either pneumococcal pneumonia or CAP caused by another pathogen. The very small number of samples is a clear limitation of our study. This pilot-study was conducted to perform a preliminary in vivo validation of the qPCR and was not designed as a full clinical trial. A larger population could have resulted in a proper cut-off value, which could be used in further studies or in clinical practice. Although the use of this limited number of OP samples was not intended to define a proper cut-off value, preliminary results are promising: best AUC of 0.714 with a sensitivity of 57.1% and specificity of 85.7% with a cutoff value of 6.000 copies/mL. The AUC was even higher when only using the samples of patients with a complete diagnostic workup; a sensitivity of 72.7% and a specificity of 84.6% using a cut-off value of 6.000 copies/mL (AUC 0.787). Choosing a different cut-off value to achieve the highest sensitivity may implicate a lower specificity. In clinical practice, in patients who are colonized with *S. pneumoniae* and infected with another bacterial pathogen, the test may be considered as (false) positive and consequently these patients would be treated with narrow-spectrum antibiotic therapy.

Recent research suggested a cut-off value of 8.000 copies/mL of the *lytA* gene when using NP swabs, which led to a sensitivity and specificity of the qPCR of 82.2% and 92% respectively[5]. The authors claim that this cut-off value is capable of differentiating between asymptomatic colonization and infection in HIV-infected patients. Another recent study used a much lower cut-off value of 102copies/mL[4].

Other researchers, who used the Spn9802 target gene found a similar cut-off value of 4.000–8.000[10].

A very recent investigation by Blake et al. used a *lytA* rt-PCR on whole blood samples to identify *S. pneumoniae* in patients with CAP in Togo. The cut-off value was set at a Ctvalue instead of the number of DNA copies/mL. The cut-off was set on a Ct-value of 35[36]. The sensitivity of the *lytA* rt-PCR was significantly higher than blood culture, 17.1% versus 12.9%, but has a much lower sensitivity compared to the *lytA* qPCR we tested on OP swabs. The specificity of the rt-PCR on blood samples was 97.4%. The authors consider this a possible consequence of cross-reactivity with *S. mitis* among other bacteria. This limitation of the *lytA* PCR has been described in other research as well. One study tested 11 streptococcal isolates that showed conflicting or previously unknown patterns when using optochin susceptibility, bile solubility, *lytA* PCR and multilocus sequence analysis and discovered that three strains were misidentified with the *lytA* rt-PCR (one false-negative result and two *S. pseudopneumoniae* strains led to a false-positive result)[22]. In three patients without detection of *S. pneumoniae* using the current diagnostic standard, but with detection of a virus (two coronaviruses and one influenza A virus) concentrations of *S. pneumoniae* above 40.000 copies/mL were detected, which limits the specificity of our test in this experiment. A possible explanation for these high concentrations of DNA copies/mL is false-negative results of the current pneumococcal tests. Only one of these patients was pretreated with antibiotics and they all had a favorable outcome with amoxicillin. Given the high DNA concentrations above the cut-off values for colonization and the favorable response to therapy an underlying pneumococcal infection seems very likely.

Previously the usefulness of the qPCR has been questioned in patients who were pre-treated with antibiotics[26,37]. The total number of patients who have been pre-treated with antibiotics in the present study is rather low (16.1%) and no reliable conclusion can be made on this topic. We believe this is an important issue not only with qPCR but with any microbiological test, so further studies should address this question[12].

A recent study showed that it is possible to detect 26 respiratory bacteria and viruses with one single test. 85% of the patients tested had been pre-treated with antibiotic therapy, and still in 78% of these patients a bacterial pathogen was detected, where only 32% of cultures were positive[38]. A bacterial pathogen was found in 71.5% of cases. No blood cultures or urinary antigen tests were included and only mucopurulent sputum was used. S. pneumoniae was detected in 35.6% of cases.

A possible explanation for the low sensitivity is the cutoff value, which at this point is based on a low number of patients, as mentioned before. When counting all positive results (every patient with a DNA copy number of more than zero) the sensitivity of the qPCR is 82% (23 out of 28 patients). Another possible explanation for the low sensitivity compared to the in-vitro sensitivity is the sample technique or sample site. Some studies use sputum samples which are difficult to obtain, whereas others use NP swabs instead of OP swabs[4,5,19]. One of these studies compared trans-nasal and transoral sampling, and concluded that the nasopharynx is the main reservoir for *S. pneumoniae*[23], but data on the best sampling technique is limited and unclear about which technique is superior. According to the WHO Pneumococcal Carriage Working Group NP samples have a slightly higher sensitivity in detecting colonization with *S. pneumoniae* in healthy adults and children. A combination of NP and OP samples is recommended for detection of *S. pneumoniae* carriage in adults. There are no current recommendations about molecular diagnostics and detection of *S. pneumoniae* in patients with CAP[39,40]. Recent research showed that in healthy adults and adults with influenza-like-symptoms the qPCR targeting *lytA* and *piaA* or *piaB* yielded more positive results than cultures (carriage in healthy adults 20% using the qPCR vs. 5% detection using cultures). The detection rate of *S. pneumoniae* in adults with influenza-like-illness was highest in saliva samples (28%) followed by OP swabs (11%), cultures (10%) and NP swabs (5%)[41,42].

Primarily, the test will have to be validated in a larger collection of clinical samples so a distinction between colonization and infection can be made. Another important question is the performance of the qPCR in patients pre-treated with antibiotics. Furthermore, the additional value needs to be determined to see if the qPCR will increase microbiological yield and leads to changes in antibiotic regimes.

In conclusion, we were able validate a quantitative PCR targeting *lytA* with good in-vitro test characteristics. One to 10 DNA copies per reaction could be detected with an in-vitro sensitivity and specificity of 100%. The results of the in-vivo tests are promising with a sensitivity of 57.1% and a specificity of 85.7%.

We believe the qPCR targeting *lytA* could be a rapid and reliable tool for diagnosing pneumococcal CAP, but further research with larger groups is necessary.

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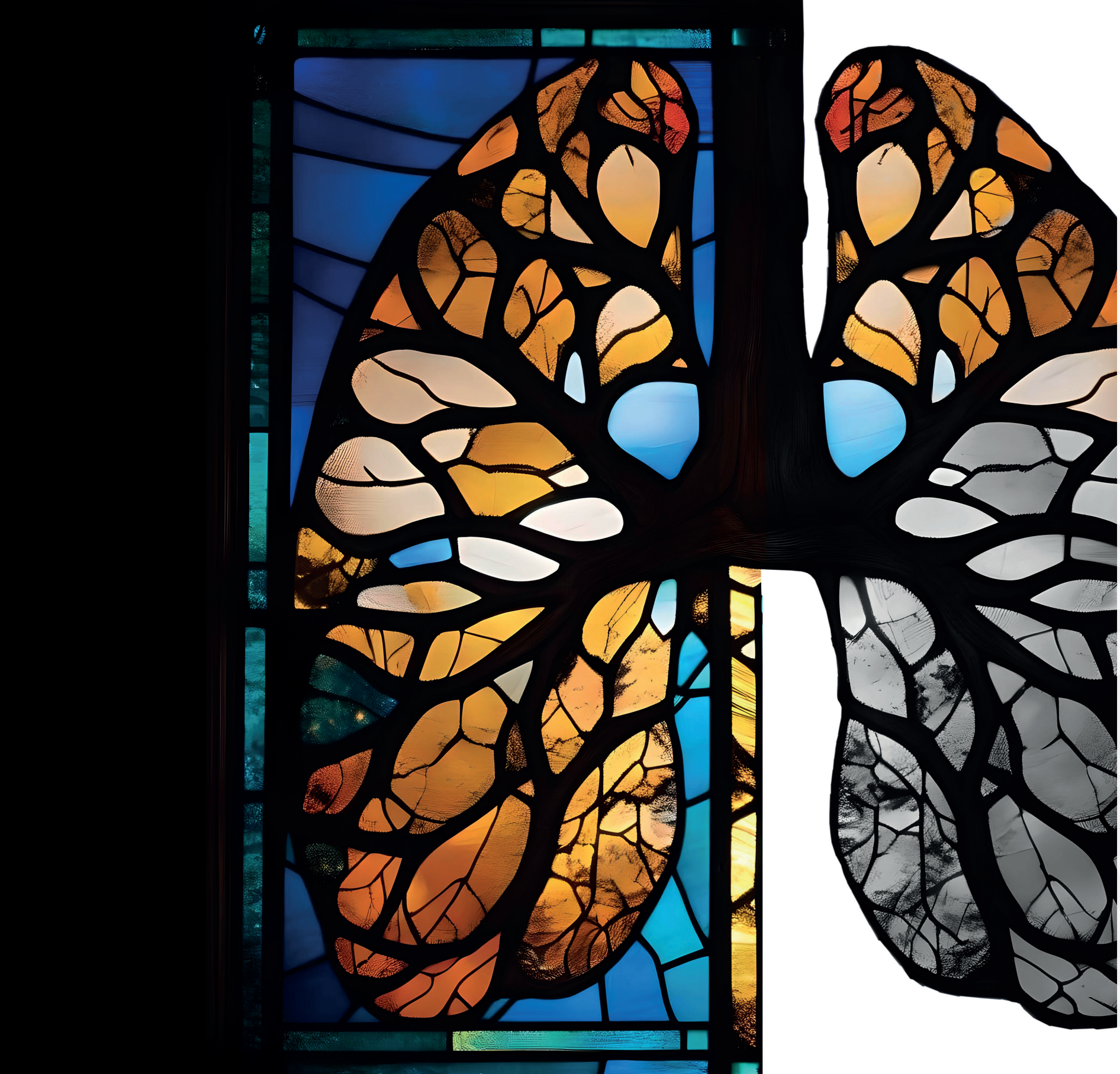

Chapter 3

Validating a clinical prediction score for Legionella related community acquired pneumonia

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BMC Infectious Diseases (2022) 22:442

ABSTRACT

Background

Legionella-related community acquired pneumonia (CAP) is a disease with an increasing incidence and a high mortality rate, especially if empirical antibiotic therapy is inadequate. Antibiotic treatment highly relies on clinical symptoms, although proven non-specific, because currently available diagnostic techniques provide insufficient accuracy for detecting Legionella CAP on admission. This study validates a diagnostic scoring system for detection of *Legionella*-related CAP, based on six items on admission (Legionella prediction score).

Methods

We included patients with *Legionella*-related CAP admitted to five large Dutch hospitals between 2006 and 2016. Controls were non-*Legionella*-related CAP patients. The following six conditions were rewarded one point if present: fever >39.4°C; dry cough; hyponatremia (Na) <133 mmol/L; lactate dehydrogenase (LDH) >225 mmol/L; C-reactive protein (CRP) >187 mg/L and platelet count <171 x 10^9/L. The accuracy of the prediction score was assessed by calculating the area under the curve (AUC) through logistic regression analysis.

Results

We included 131 cases and 160 controls. A score of 0 occurred in non-*Legionella*-related CAP patients only, a score of 5 and 6 in *Legionella*-related CAP patients only. A cut-off ≥4 resulted in a sensitivity of 58.8% and a specificity of 93.1%. The AUC was 0.89 (95%CI 0.86-0.93). The strongest predictors were elevated LDH, elevated CRP and hyponatremia.

Conclusion

This multi-centre study validates the Legionella prediction score, an easily applicable diagnostic scoring system, in a large group of patients and finds high diagnostic accuracy. The score shows promise for future prospective validation and could contribute to targeted antibiotic treatment of suspected *Legionella* CAP.

INTRODUCTION

Legionella infection is an important cause of community-acquired pneumonia (CAP) with a mortality of 8-12%[1]. The average incidence of Legionella infection in CAP was reported 2.1-3.6% in a recent meta-analysis[2]. However, due to underdiagnosis, the true incidence is probably higher. In the US, the incidence of reported Legionella cases quadrupled over the past decades[3–6]. *Legionella*-related pneumonia has an overall higher burden of morbidity and mortality than other causes of CAP, especially if initial empirical antibiotic therapy is inadequate[3,5,7,8].

Diagnosis of Legionella-related CAP is difficult, because culturing Legionella from sputum and blood takes tree to ten days and has a low yield. The introduction of the urinary antigen test (UAT) for *Legionella pneumophila* improved diagnosis, especially in severe cases. However, the UAT can be negative in the early phase of the disease, especially in patients with mild disease. UAT detects only Legionella pneumophila serogroup 1 antigens, accounting for more than 80% of *Legionella* cases[9–11]. Over the last years several other subspecies of Legionella have been associated with significant clinical disease, especially Legionella longbeachae which is predominantly found in Australia, New Zealand and Asia, but recently has been detected in both USA and Europe[12–14].

To prevent overuse of macrolides and quinolones, international guidelines recommend empirical antibiotic coverage of Legionella only when this infection is suspected based on clinical signs and symptoms, or in patients with severe CAP. Clinical scoring systems were developed to predict *Legionella*-related pneumonia, but most have limited clinical significance because of low accuracy or the need to include follow-up data over several days[15–17]. As a result initial empirical coverage may be inadequate[18–20].

Fiumefreddo et al.[21] developed a diagnostic scoring system consisting of 6 items which are easily obtainable on admission, namely fever, dry cough, hyponatremia, elevated lactate dehydrogenase (LDH) and elevated C-reactive protein (CRP), further called: Legionella prediction score. In the derivation cohort, the diagnostic accuracy of the score was high, with an area under the curve (AUC) of 0.86 (95% confidence interval (CI) 0.81-0.90)[21]. In a previous validation study with 37 cases, the Legionella prediction score discriminated reasonably well between Legionella-related CAP and CAP caused by other pathogens (specificity 92% and sensitivity 31% at cut off ≥4, area under the curve 0.91)[22].

In theory this prediction score could be a useful clinical tool to limit antibiotic overuse in selected patients. Therefore, we evaluated the performance of this score in a large, Dutch cohort of patients with Legionella-related CAP.

METHODS

Patients and materials

In this cross-sectional, observational, retrospective study, data was collected from four large teaching hospitals and one University hospital in the Netherlands. A list of all patients tested positive with Legionella species between 2006 and 2016 was provided by the departments of microbiology. Medical records of all patients were reviewed and data was collected anonymously. Cases had at least one microbiological test positive for *Legionella* species, either culture, serology, PCR or UAT. Our control group consisted of non-*Legionella* CAP-patients who required hospital admission, through random selection of participants from the REDUCE study, which was conducted in the Netherlands from 2013-2017 (full study protocol available via clinicaltrials.gov, NCT01964495).
All patients included in the study had at least one consolidation on the chest X-ray together with clinical signs and symptoms indicative for CAP. Patients were excluded if they were pregnant or breastfeeding, if they had immunodeficiency or cancer, in case of obstruction, aspiration or hospital acquired pneumonia, if they could not follow the REDUCE protocol and if items needed to calculate the Legionella prediction score were missing. No permission by the medical ethical board was required for this retrospective study.

Data and Legionella prediction score

The collected data included vital parameters, clinical signs and laboratory findings on admission, relevant comorbidities, smoking history and CURB-65 score. The Legionella prediction score, ranging from 0-6, was calculated. For the six following conditions, if present, one point was scored: fever >39.4°C; dry cough; hyponatremia (sodium) <133 mmol/L; LDH >225 mmol/L; high CRP >187 mg/L and low platelet count <171 x 109/L.

Outcomes

The primary outcome was the diagnostic accuracy of the Legionella prediction score for *Legionella*-related CAP. Furthermore, we assessed the predictive value of the original, continuous parameters and the proposed cut-off points, both univariate and multivariate.

Statistical analysis

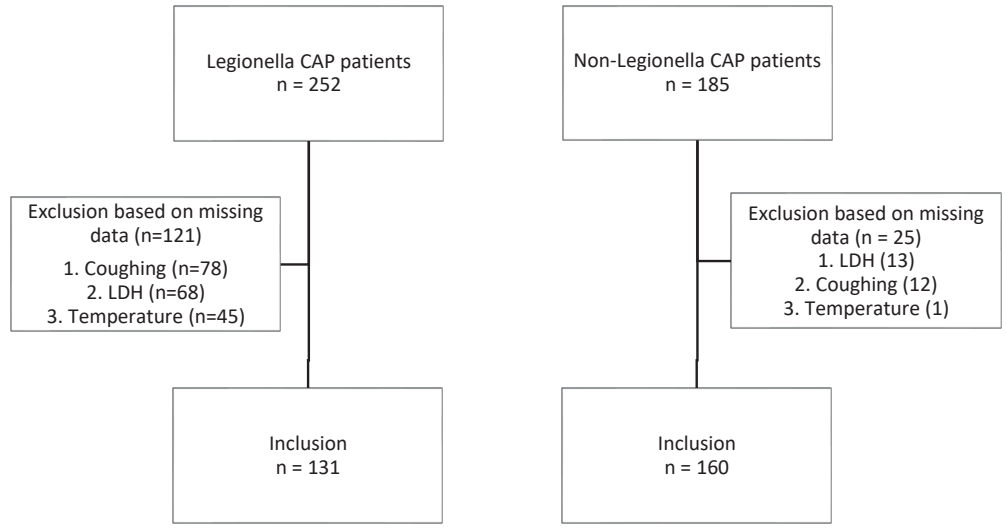
Patient characteristics were assessed for normal distribution with the Kolmogorov-Smirnov test. Either mean, standard deviation and chi-square test or median, percentiles and Mann-Whitney-U test were reported.

Continuous parameters were analysed in a logistic regression model that was performed for each individual parameter and for all parameters combined. Thereafter, parameters were dichotomized in the categories used in the prediction score. Univariate and multivariate logistic regression was repeated with these dichotomized parameters.

For each regression, the b-coefficient, the odds ratio, AUC and p were calculated. A p-value <0.05 was considered significant. Sensitivity, specificity, positive and negative predictive values were calculated for scores 0 – 6 of the prediction score.

To further assess accuracy, chi-square, the loglikelihood ratio and Nagelkerke square were calculated. IBM SPSS Statistics version 25.0 was used for all analyses.

Figure 3.1 Flow chart of inclusion and exclusion.



Patients were excluded if items needed to calculate the Legionella prediction score could not be obtained. These items are: temperature, dry cough, sodium, LDH, CRP and platelets. *CAP* community acquired pneumonia, *LDH* lactate dehydrogenase, *CRP C*-reactive protein

Table 3.1 Patient characteristics

	Legionella CAP n = 131	Non-*Legionella* CAP n = 160	p
Male, n (%)	88 (67.2)	89 (55.6)	0.037
Age [years], median (IQR)	63.4 (56.2–70.3)	72.2 (64.7–81.4)	< 0.001
Current smoker, n (%)	43 (53.8)	29 (20.1)	< 0.001
Comorbidities, n (%)			
COPD	11 (8.4)	47 (29.4)	< 0.001
Congestive heart failure	11 (8.4)	21 (13.1)	0.22
Neurologic disease	15 (11.5)	37 (23.1)	0.012
Cancer	8 (6.1)	33 (20.6)	< 0.001
Renal disease	4 (3.1)	15 (9.4)	0.033
Liver disease	4 (3.1)	2 (1.3)	0.271
Symptoms, n (%)			
Dyspnea	79 (68.7)	124 (83.8)	0.005
Dry cough	77 (58.8)	50 (31.3)	< 0.001
Headache	31 (66.0)	14 (32.6)	< 0.001
Muscle or joint pain	37 (64.9)	25 (51.0)	0.143
Nausea	36 (52.9)	28 (48.3)	0.538
Vomiting	28 (41.8)	20 (37.0)	0.686
Diarrhea	42 (53.2)	13 (15.5)	< 0.001
Confusion	24 (18.3)	32 (20.0)	0.718
Physical findings, median (IQR)			
Heart frequency [BPM]	100 (82–112)	96 (83–111)	0.635
Systolic blood pressure (mmHg)	130 (118–145)	135.5 (119–151)	0.139
Diastolic blood pressure (mmHg)	73 (65–81)	75.5 (67–83)	0.111
Body temperature (°C)	39(37.8–39.7)	38.4 (37.5–39.1)	0.002
CURB-65 [score 0–5], median (IQR)	1 (0–2)	2 (1–2)	0.016
Previous antibiotic treatment, n (%)	39 (33.6)	42 (26.8)	0.226
Laboratory findings, median (IQR)			
Urea (mmol/L)	7.1 (5.5–11.4)	7.2 (5.0–9.1)	0.333
Creatinine (mc mol/L)	101 (80–131)	88 (66–108)	< 0.001
Sodium (mmol/L)	132 (129–135)	136 (133–138)	< 0.001
Potassium (mmol/L)	3.9 (3.5–4.2)	3.9 (3.6–4.3)	0.186
Bilirubin (μmol/L)	12 (8–19)	13 (8–16)	0.628
ASAT (IU/L)	49 (33–103)	26 (19–35)	< 0.001
ALAT (IU/L)	36 (24–67)	19 (13–29)	< 0.001
LDH (IU/L)	465 (324–609)	198 (168–238)	< 0.001
AP (IU/L)	79 (69–115)	82 (67–113)	0.242
GGT (IU/L)	47 (30–79)	37 (24–67)	0.034
C-reactive protein (mg/L)	317 (244–390)	162 (77–260)	< 0.001
Hemoglobin (mmol/L)	8.3 (7.8–9.1)	8.1 (7.3–8.9)	0.067
Platelets ($\times 10^9$/L)	204 (156–249)	221 (179–293)	0.002
White blood cell count ($\times 10^9$/L)	13 (10–17)	13 (10–18)	0.377

CAP community acquired pneumonia, *IQR* interquartile range, *BPM* beats per minute, *ASAT* aspartate transaminase, *ALAT* alanine transaminase, *LDH* lactate dehydrogenase, *AP* alkaline phosphatase, GGT gamma-glutamyltransferase

RESULTS

We identified 252 patients with Legionella-related CAP and 185 patients with non-Legionella related CAP. Of 252 patients with Legionella, 131 had complete data and were included. Of non-Legionella patients, 160 were included as controls (Fig. 3.1). Baseline characteristics are summarized in Table 3.1. Patients with Legionella were often male, relatively younger, had less comorbidities (such as COPD and cancer), but were more frequently active smokers.

Of cases, 126 (96%) were confirmed by UAT and 29 (22%) were confirmed by sputum PCR or culture. In the control group, the most frequently detected pathogens were Streptococcus pneumoniae (18.8%), Staphylococcus aureus (10.1%) and Haemophilus influenzae (8.1%). A further specification of pathogens in the control group is available in Table 3.2.

In univariate regression of the original values (Table 3.3), all six predictors were significantly associated with Legionella-related CAP. The strongest predictors were sodium, CRP and LDH levels (AUC respectively 0.76, 0.80 and 0.93). In multivariate regression, this association persisted for all parameters except for dry cough.
The AUC of the multivariate model of these variables was 0.96 (95% CI 0.94–0.98).
In Table 3.4 all variables were expressed as dichotomous parameters. In univariate analysis again, all predictive values were statistically significant. The strongest predictors were hyponatremia < 133 mmol/L, elevated CRP > 187 mg/L and elevated LDH > 225 mmol/L (AUC respectively 0.71, 0.75 and 0.81). In the multivariate model, dry cough was a significant predictor. Fever above 39.4 °C and platelets below 171 × 109/L were not significant predictors. The AUC of the complete dichotomic multivariate model was 0.89 (95% CI 0.86–0.93).

As shown in Fig. 3.2, a prediction score of 0 only occurred in non-Legionella-related CAP patients. Above, the number of cases gradually increased per score point.
A prediction score of 5 or 6 points was only found in Legionella-related CAP patients (specificity 100%). The prediction score detected Legionella with a specificity of 93.1% and a sensitivity of 58.8% when a cut-off ≥ 4 was chosen. A cut-off ≥ 2 resulted in a sensitivity of 98.5% and a specificity of 50.6%. Figure 3.3 illustrates the receiver operating characteristics curve (ROC-curve) of the individual predictors and of the prediction score.

Table 3.2 Pathogens detected in participants with non-Legionella community acquired pneumonia

Pathogen	n	(%)
S. pneumoniae	30	(18.8)
S. aureus	16	(10.0)
H. influenzae	13	(8.1)
Influenza A Virus	11	(6.9)
E. coli	9	(5.6)
M. pneumoniae	7	(4.4)
P. aeruginosa	7	(4.4)
M. catarrhalis	5	(3.1)
Rhinovirus	4	(2.5)
K. pneumoniae	4	(2.5)
Coronavirus	3	(1.9)
H. parainfluenzae	2	(1.3)
Other	6	(3.8)
None	74	(44.6)

Table 3.3 Univariate and multivariate analysis of the different predictors

	Univariate analysis			Multivariate analysis		
	B	OR (95% CI)	p	B	OR (95% CI)	p
Temperature	0.29	1.33 (1.08–1.64)	0.007	0.508	1.66 (1.15–1.64)	0.007
Dry cough	1.14	3.14 (1.94–5.08)	<0.001	0.640	1.90 (0.83–5.08)	0.128
Sodium	−0.215	0.81 (0.76–0.86)	<0.001	−0.144	0.87 (0.79–0.86)	0.002
LDH	0.016	1.02 (1.01–1.02)	<0.001	0.015	1.02 (1.01–1.02)	<0.001
CRP	0.009	1.01 (1.01–1.01)	<0.001	0.008	1.01 (1.00–1.01)	<0.001
Platelets	−0.005	1.00 (0.99–1.00)	0.002	−0.005	1.00 (0.99–1.00)	0.046

	AUC (95% CI)
Temperature	0.61 (0.54–0.67)
Dry cough	0.64 (0.57–0.70)
Sodium	0.76 (0.70–0.81)
LDH	0.93 (0.90–0.96)
CRP	0.80 (0.75–0.85)
Platelets	0.61 (0.54–0.67)

OR odds ratio, *CI* confidence interval, *AUC* area under the curve, *LDH* lactate dehydrogenase, *CRP* C-reactive protein

Table 3.4 Univariate and multivariate analysis of the dichotomized variables

	Univariate analysis			Multivariate analysis		
	B	OR (95% CI)	p	B	OR (95% CI)	p
Temperature > 39.4 °C	0.77	2.17 (1.24–3.78)	0.006	0.45	1.56 (0.66–3.70)	0.311
Dry cough		*See Table 3*		0.82	2.28 (1.10–4.73)	0.027
Sodium < 133 mmol/L	1.84	6.27 (3.72–10.58)	<0.001	1.44	4.24 (1.98–9.08)	<0.001
LDH > 225 mmol/L	3.74	42.1 (17.4–101.7)	<0.001	3.42	30.54 (11.3–82.4)	<0.001
CRP > 187 mg/L	2.64	14.0 (7.3–26.9)	<0.001	2.23	9.29 (4.11–21.03)	<0.001
Platelets < 171 x 10^9/L	0.56	1.76 (1.03–3.01)	0.039	0.70	2.02 (0.88–4.65)	0.099

	AUC (95% CI)
Temperature > 39.4 °C	0.57 (0.50–0.63)
Dry cough	
Sodium < 133 mmol/L	0.71 (0.64–0.77)
LDH > 225 mmol/L	0.81 (0.76–0.86)
CRP > 187 mg/L	0.75 (0.70–0.81)
Platelets < 171 x 10^9/L	0.55 (0.49–0.62)

OR odds ratio, *CI* confidence interval, *AUC* area under the curve, *LDH* lactate dehydrogenase, *CRP* C-reactive protein

Figure 3.2 Distribution of participants per score.

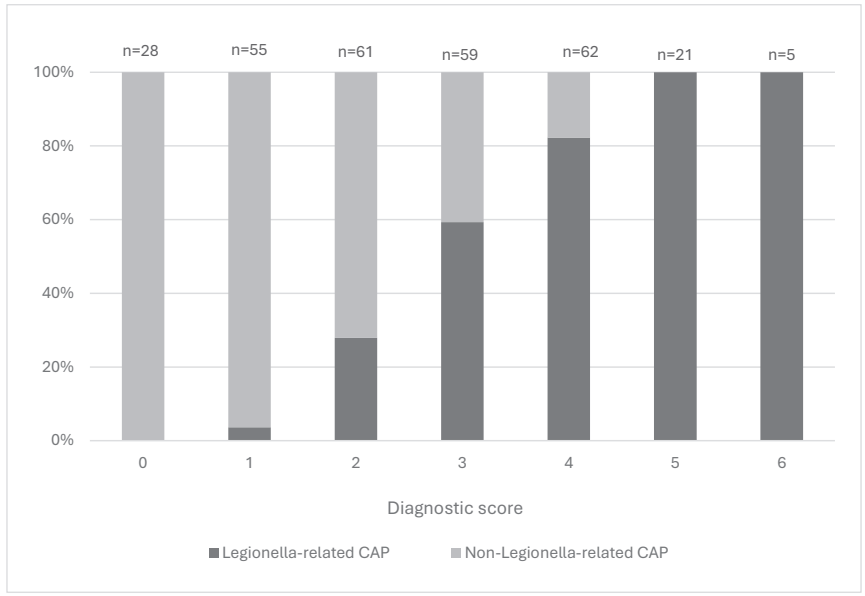
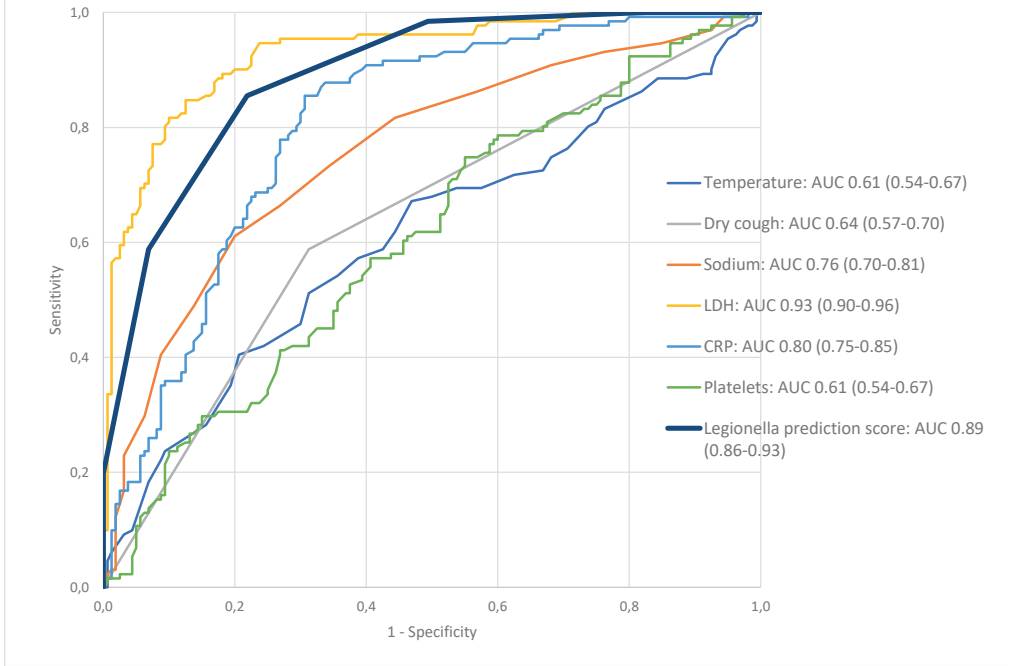


Figure 3.3 ROC-curve of individual parameters and Legionella predictive score.



This figure shows the ROC-curve of the individual parameters, analyzed as continuous values. Furthermore, it shows the ROC-curve of the diagnostic scoring system, which is calculated by dichotomizing the individual parameters, followed by multivariate regression analysis. *ROC-curve* receiver operating curve, *AUC* area under the curve, *LDH* lactate dehydrogenase, *CRP* C-reactive protein

DISCUSSION

Legionella-related CAP is a disease with a high mortality rate and increasing incidence[1–6]. It requires targetedantibiotic treatment, in an era where antibiotic resistance is rising and antibiotic stewardship is important. Although clinical symptoms of Legionella prove non-specific[15], they can be a decisive factor in the treatment choice on admission[18–20]. This retrospective study further validated a prediction score based on six clinical parameters, that can be applied easily on admission, and found a high accuracy with an AUC of 0.89 (95% CI 0.86-0.93). We demonstrated that this score can potentially be used to rule-in or rule-out Legionella CAP, depending on the cut-off point chosen. Therefore, in patients presenting with mild to moderate disease symptoms, it could be applied both for early identification and specific treatment of those infected with Legionella, in particular in cases that are not detected by UAT. The negative predictive value of the score will likely be higher in an unselected population of hospital admitted CAP patients, since the incidence of Legionella is lower than in our population.

All predictors were associated significantly with the outcome. However, temperature and platelets were no significant predictors in the multivariate analysis after dichotomization. Assumably, this can be explained by the wide range in which these variables occurred in both patients with Legionella CAP and with non-Legionella CAP.

Our study yielded an accuracy similar to that found by a Spanish study (AUC 0.86 (95% CI 0.81–0.90)), based on 82 cases[21]. It was higher than in a previous multinational validation study, which found an AUC of 0.73 (95% CI 0.65–0.81)[22]. This difference can be explained by a smaller sample size (37 cases). Baseline differences between the cases and controls (age, COPD and smoking) in our study were similar to both other studies[22]. This was not the case in a Japanese validation study published in 2017, in which participants were more often male and that also included patients with cancer[24]. They found a sensitivity of 94% and a specificity of 49% at a cut-off ≥ 2, resembling our present study.

In the literature two other diagnostic scoring systems for Legionella-related CAP were proposed, namely the Winthrop University score and the Community-Based Pneumonia Incidence Study Group scoring system. These two scoring systems were validated, but found unsuitable for diagnosing or excluding Legionella in a clinical setting, due to low accuracy[16,17,24,25].

A Japanese study group recently proposed a variation on the Legionella prediction score, which includes dyspnoea and gender instead of on temperature and platelets.
This score performed well (AUC 0.93) in a Japanese validation cohort. However, in study populations outside Japan, male gender and dyspnoea were not identified as risk factors for Legionella-related CAP. Therefore this score may be less relevant[26].

This multi-centre study included a large number of patients with Legionella-related CAP. The number of participants considerably exceeds the number that is due sufficient for validation of a prediction score with a dichotomous outcome, according to Toll et al[23]. All hospital admitted patients with CAP were eligible for inclusion and data was col-

lected from five different large hospitals with a wide geographical spread. This adds to the external validity of the study because it closely resembles a real-life clinical population. We chose to only include patients with complete data, so imputation of missing data could be avoided which adds on to the internal validity of the study. However, this has the potential to introduce some sort of selection bias but given the large sample of patients we believe the effect of this potential bias is likely small.

A weak point of this study is that its retrospective. Missing data on occurrence of especially (dry) cough lead to many exclusions. In a prospective study setting, this parameter would be easy to obtain. Furthermore, cases were retrospectively selected, based on positive microbiological tests. Mostly, this was the UAT, which does not detect species other than Legionella pneumophila serogroup 1. Because cultures and PCR have not been performed in all participants, some Legionella cases might have been missed. This could potentially influence the performance of the score. A Japanese study demonstrated a better performance of the Legionella prediction score for Legionella serogroup 1 (N = 11) than for other Legionella species (n = 23)[27]. This suggests that the score is particularly useful for detecting Legionella serogroup 1, which was detected in 96% of the cases in the present study.

Future research should validate the diagnostic scoring system prospectively, preferably in an unselected CAP population, in which Legionella is detected via UAT, PCR and cultures. This research could also analyse the accuracy of the scoring system, give more insight into performance of the score over the course of the disease, mild versus advanced disease, and investigate its clinical significance in addition to UAT. Moreover, longitudinal studies on clinical outcomes resulting from implementation of the test, such as change in antibiotic prescriptions, mortality, ICU admissions and of length of stay in the hospital, are needed.

CONCLUSION

This six-items prediction score detects Legionella related CAP infections with a high specificity of 93.1% (sensitivity 58.8%) in patients who score positive for at least four items. It is easy to implement in day to day practice with data readily available in every CAP patient and. Overall, based on our data and previous studies we believe it shows promise for further prospective validation and could contribute to targeted antibiotic treatment of Legionella-related CAP.

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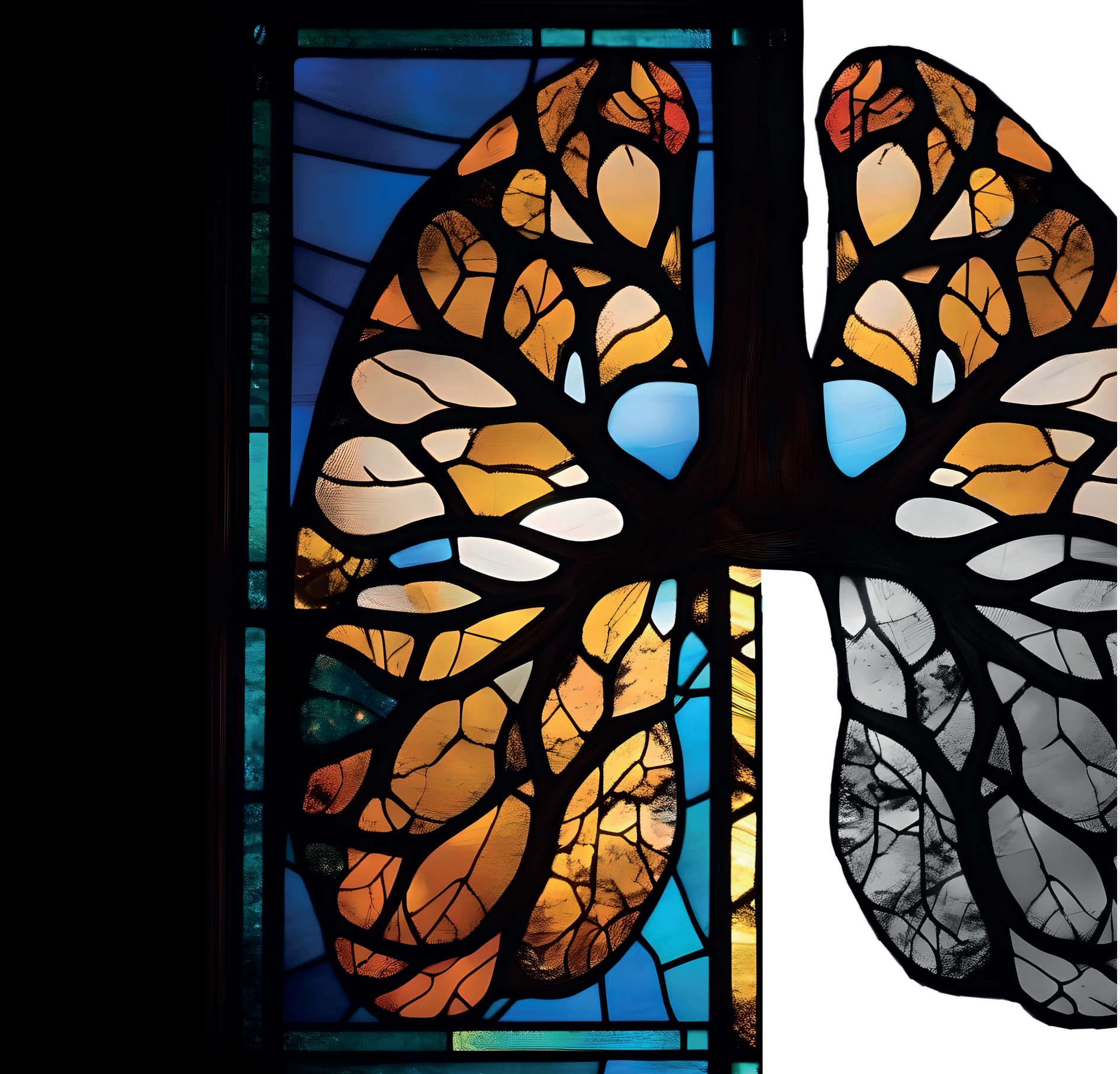

Chapter

4

Plasma cytokine profile on admission related to aetiology in Community Acquired Pneumonia

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ABSTRACT

Background

Potentially unnecessary antibiotic use for community - acquired pneumonia (CAP) contributes to selection of antibiotic-resistant pathogens. Cytokine expression at the time that treatment is started may assist in identifying patients not requiring antibiotics. We determined plasma cytokine patterns in patients retrospectively categorized as strict viral, pneumococcal or combined viral - bacterial CAP.

Objective

To investigate whether cytokine - based prediction models can be used to differentiate strict viral CAP from other aetiologies at admission.

Methods

From 344 hospitalized CAP patients, 104 patients were categorized as viral CAP ($n = 17$), pneumococcal CAP ($n = 48$) and combined bacterial-viral CAP ($n = 39$). IL-6, IL-10, IL-27, IFN-γ and C-reactive protein (CRP) were determined on admission in plasma. Prediction of strict viral aetiology was explored with two multivariate regression models and ROC curves.

Results

Viral pneumonia was predicted by logistic regression using multiple cytokine levels (IL-6, IL-27 and CRP) with an AUC of 0.911 (95% CI: 0.852-0.971, P < .001). For the same patients the AUC of CRP was 0.813 (95% CI: 0.728 - 0.898, P < .001).

Conclusions

This study demonstrated differences in cytokine expression in selected CAP patients between viral and bacterial aetiology. Prospective validation studies are warranted.

INTRODUCTION

Of all infectious diseases, community - acquired pneumonia (CAP) is the number one cause of death in the developed world and is often empirically treated with antibiotics[1-3]. Despite the use of several time - consuming microbial and molecular techniques, a conclusive microbiological diagnosis is only established in up to 50% of patients presenting with CAP[4-7]. Respiratory viruses can be identified by polymerase chain reaction (PCR) in 20% - 40% of CAP patients[6]. If respiratory pathogens are detected by PCR, these viral agents can be causative or non - causative for infection. When causative, they can be in fact coinfections with undetected bacterial pathogens or strict viral infection. 'Strict viral' CAP is defined as when a respiratory virus is the only causative pathogen for CAP in a patient. For strict viral CAP, antibiotics are probably ineffective and in theory should be withheld.

Symptom - based prediction of aetiology has proven inadequate to discriminate between viral and bacterial aetiology[8-11]. An alternative strategy to predict bacterial aetiology is the use of biomarkers like C - reactive protein (CRP) or procalcitonin (PCT)[12,13]. High (>0.5 mcg/L) PCT values are used to initiate the use of antibiotics and low values (<0.1 mcg/L) strongly discouraged the use of antibiotics. However, this strategy fails to distinguish viral from atypical pathogens and thus cannot be used to identify those patients in which antibiotics can be withheld[14].

In the early phase of pneumonia, bacteria and viruses trigger distinct innate immune response pathways. As a consequence, several differentiating inflammatory mediators are likely to be markedly elevated and could serve as potential biomarkers. In bacterial CAP, rapid interleukin-17A(IL-17A) production by gamma-delta T cells attracts, expands and activates neutrophils at the site of infection[15]. Release of young neutrophils from the bone marrow is an innate response aimed at mainly extracellular pathogens, such as pneumococci[16]. IL-6 enhances general pro-inflammatory activity as well as T-helper-17 (Th-17) development from naive T cells[17]. In bacterial pneumonia higher levels of IL-6, TNF-α and interleukin-1 (IL-1) were found in bronchoalveolar lavage (BAL) compared to healthy controls. Also in serum, IL-6 was elevated in bacterial CAP compared to healthy individuals[18]. A certain 'spill' of cytokines or a systemic response might be responsible for this finding.

In viral CAP, type 1 interferons (IFN) are produced by infected cells, often airway epithelial cells. Natural Killer (NK) cell and also CD8 T cells will produce type 2 interferon (IFN-γ) in response to viral replication. Interferons limit viral replication and enhance the T-helper-1 pathway response. However, T-helper-2 pathway cytokines, such as IL-5, results in the recruitment and activation of eosinophils, which can display anti-viral activities as well[19,20,21].

In mixed bacterial/viral infections, primary viral pathogens can enhance bacterial infection. Adherence of bacteria to epithelial cells is enhanced in virus-infected cells, predisposing to superinfection[22], but several alternative mechanisms have been proposed. CAP patients with primary influenza infection have elevated levels of IL-27, which in

turn inhibit IL-10 production and therewith the Th-17 pathway. This does not alter viral clearance, but limits lung neutrophil influx and potentially diminishes bacterial clearance[23-25].

In the present study, we investigated whether plasma cytokine levels of the Th17, Th1 and Th2 pathways can be used to distinguish between three aetiological groups: pneumococcal, viral and mixed viral/bacterial infection. First, we investigated absolute cytokine plasma-level differences between groups. Hereafter, we investigated whether cytokine-based prediction models can be used to differentiate viral CAP from other aetiologies at admission, and whether this adds value to the routine determination of CRP.

METHODS

Patients

In the present study, patients were admitted to the Northwest Hospital group Alkmaar with CAP from October 2013 to September 2016. Patients were part of the REDUCE trial, which was a randomized controlled multicentre trial in patients admitted to a regular hospital ward with radiologically proven CAP (NCT01964495). All data were prospectively collected in a standardized manner. Blood samples, blood cultures for aerobic and anaerobic pathogens, oropharyngeal swabs for respiratory viruses and atypical pathogens, urine antigen tests for *Legionella pneumophila serotype 1* and *S. pneumoniae* and if possible, sputum cultures were obtained for all patients. The regional ethical committee approved the REDUCE trial, and the re-use of samples for this retrospective studies.

Inclusion and exclusion criteria

Patients were eligible for inclusion in the study if they met the following inclusion criteria: age ≥18, need for hospitalization and a life expectancy >30 days, informed consent was obtained from either the patient or their legal representative. All patients had to have new consolidation(s) on the chest radiograph and a clinical presentation of an acute illness with one or more of the following symptoms: temperature ≥38.0°C (100.4°F), dyspnoea, cough (with or without expectoration of sputum), chest pain, malaise or fatigue, myalgia, gastrointestinal symptoms, rales, rhonchi or wheezing, egophony or bronchial breath sounds and haemoptysis. Exclusion criteria were: severe immunosuppression (eg, HIV infection, chemotherapy), active neoplastic disease, obstruction pneumonia (eg, in lung cancer), aspiration pneumonia, pneumonia that developed within 8 days after hospital discharge, inability and/or unlikeliness to comprehend and/or follow the protocol, pregnancy and/or lactation.

Categories of CAP

We defined three aetiological groups based on pathogens demonstrated by routine microbiological procedures. Patients in which no pathogens were detected or patients that did not match the criteria for one of the three groups were excluded from the analysis (Figure 4.1). Groups were defined as: strict viral CAP (n = 17); oropharyngeal swab PCR-positive for a virus, PCT levels on admission ≤0.25 µg/L and no bacteria detected. Adenoviruses and rhinoviruses were excluded from this group as they are generally not considered to be virulent enough to cause CAP in this sample of adult immunocompetent patients with community-based aetiology. We believe that patients having a sole adenovirus or rhinovirus sampled, cannot be stated as viral CAP with enough certainty to include. Pneumococcal CAP (n = 48); pneumococci detected by any microbial technique (blood, sputum, urine antigen test) and no other pathogens identified. Mixed CAP (n = 39): defined by the presence of at least one bacterial species plus the presence of at least one viral species (other than adenovirus and rhinovirus) by PCR, and no fungi or yeasts detected.

Baseline and outcome measurements

Baseline characteristics comprised age, gender, co-morbidity according to the Charlson index, smoking status (current/non-current), chest radiograph localization of consolidation (unilobar, multilobar, bilateral). CURB-65 scores validated to predict short-term mortality for CAP were calculated. Primary outcome variables consisted of the following cytokines in picogram per millilitre (pg/mL): IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17A, IL-21, IL-22, IL-23, IL-27, TNF-α, IFN-γ and IP-10. Secondary outcome variable was the CRP serum level upon admission in milligram per litre (mg/L).

Cytokines assay

Luminex assay (eBioscience, ProcartaPlex) was used to measure cytokines plasma levels, which were read on a-Bioplex 200 (BioRad). Samples were collected and processed in a standardized manner on admission and stored at -80°C at the laboratory of the Northwest Hospital till analyses at the Academical Medical Centre of Amsterdam. Samples had not been thawed before.

Statistics

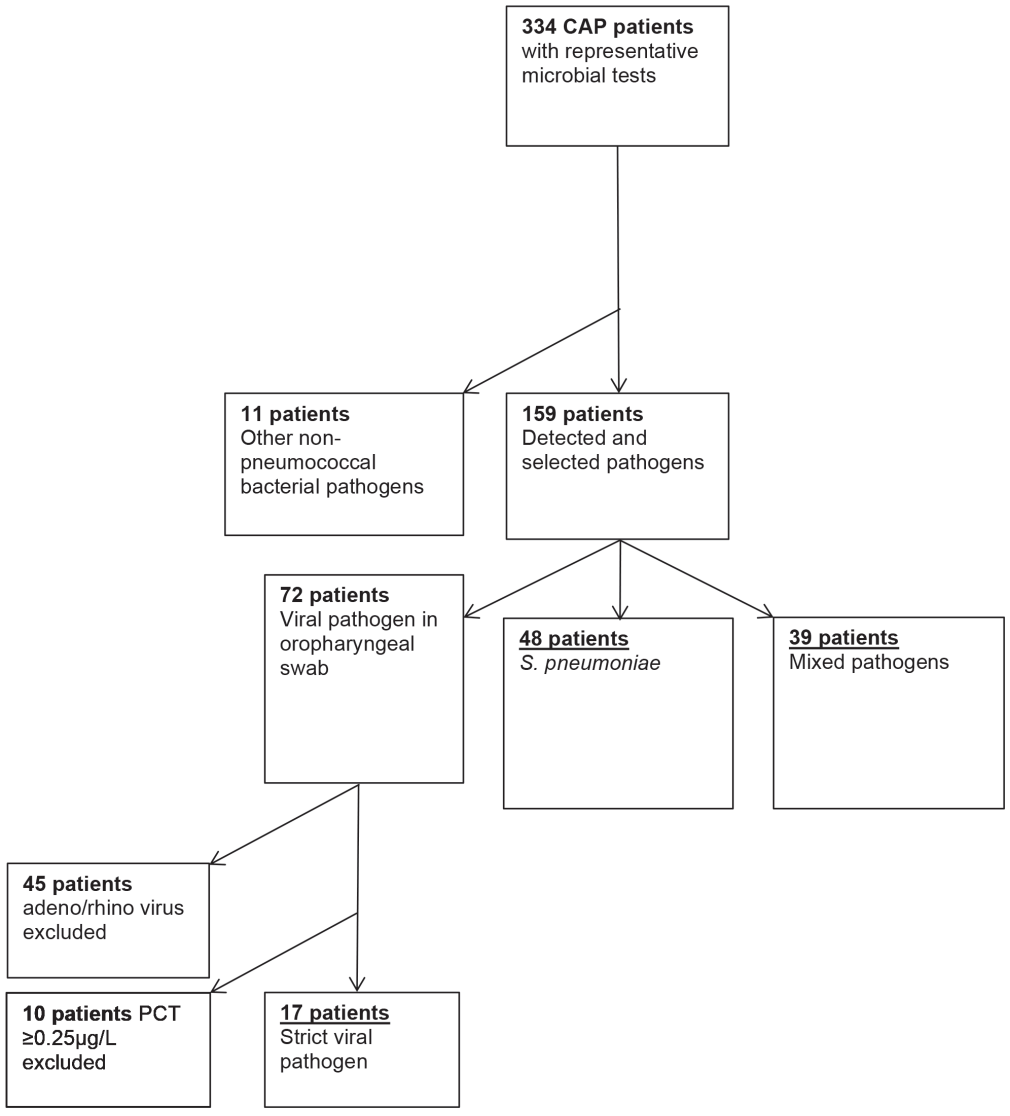
Descriptive statistics are used to compare baseline characteristics between strict viral, pneumococcal and mixed CAP. Continuous variables are presented as mean or median with standard deviation or IQR depending on the normality of the distribution and categorical variables as proportions. Differences in cytokine levels between the three groups were tested with the Kruskal-Wallis test, followed by Mann-Whitney tests between groups where appropriate. For the prediction model, we compared the strict viral CAP with the remaining CAP patients (pneumococcal CAP and mixed CAP). First, we constructed a model containing only cytokines, then containing only CRP and then compared this to a model with cytokines and CRP together. Last, we created two models on a population without selection by PCT.

We performed multivariate logistic regression with backward selection using the Wald statistic with a *P* value of .1, starting with all cytokines and adding CRP as a fixed variable. The predicted probabilities for having strict viral CAP were calculated for each individual subject, and analysed with ROC curves. Linearity of cytokines to the log odds of the

outcome were assessed using Box-Tidwell tests. IBM SPSS Statistics for Windows, version 24.0 was used for all analyses. A P value below .05 was considered statistically significant for all data but the selection of variables in the prediction models.

RESULTS

Figure 4.1 Numbers of patients per group



Final three groups are shown underlined. Patients in which no pathogen was detected, patients with nonpneumococcal bacterial aetiology, single adeno/rhinovirus infected patients were excluded from analysis. Viral CAP patients with PCT ≥ 0.25 µg/L (n = 10) were excluded because of possible coinfection with bacterial pathogen(s)

The demographics of the three groups did not reveal statistically significant differences in gender, smoking status, comorbidities and disease severity (Table 4.1). All groups contained comparable rates of patients with chronic obstructive pulmonary disease (COPD). The various pathogens per aetiological group are listed in Table 4.2.

There were significant differences in median plasma levels of IL-6, IL-10, IL-17A and IFN-γ between the three aetiologies (Table 4.3). IFN-γ was elevated in viral CAP patients. IL-6 was elevated in pneumococcal CAP. These two cytokines were significantly different in all three groups even though there was still some overlap as depicted in Appendix Figure SA1. In order to explore if prediction of aetiology was possible, we created two prediction models using multivariatelogistic regression: the first model consisted only of the measured cytokines, the second one also included CRP (Table 4.4). In the models $(IFN\text{-}\gamma)^2$ was used because IFN-γ was collinear with the outcome, all other cytokines were not.

To prove the clinical use of cytokine measurements over CRP alone, we compared to an AUC of CRP alone, which has an AUC of 0.813 (95% CI: 0.728-0.898, P < .001) (figure SA2). ROC curves were created for both models (Figure 4.2). IL-5 and IL-27 contributed to the models, although these cytokines were non-significant as single predictors of aetiology.

In a cytokine-alone model, IL-5, IL-6 and IFN-γ appeared to distinguish viral from bacterial CAP (ie, pneumococcal and mixed CAP together) best. In this first model, 35% of the variance in subjects either having strict viral or other aetiology CAP, can be explained by the cytokines IL-5, IL-6 and IFN-γ (R2 = .353). Figure 4.2 shows the diagnostic accuracy to differentiate between strict viral and bacterial-induced CAP. The cytokine-based model calculates the chance of viral CAP for each CAP patient. We created an AUC curve with an area under the curve of 0.863 (95% CI: 0.768-0.958, P < .001). Using a cut-off value of 0.65, there is 99% specificity and 18% sensitivity.

Table 4.1 Baseline variables

	Strict viral (n=17)	*Pneumo coccal (n=48)*	*Mixed Infection (n=39)*
Age in years	71 (17)	68 (15)	67 (19)
Male gender	7/17 (41%)	28/48 (58%)	17/39 (44%)
Median Charlson index	1	1	0
COPD	2/17 (12%)	17/48 (35%)	10/39 (26%)
Current smoker	2/17 (12%)	14/45 (31%)	9/39 (23%)
Consolidation			
unilobar	10/15 (67%)	39/47 (83%)	26/34 (76%)
multilobar	5/15 (33%)	8/47 (17%)	8/34 (24%)
bilateral	1/15 (7%)	7/47 (15%)	5/34 (15%)
CURB-65 score			
1	3/17 (18%)	12/48 (25%)	14/39 (36%)
2	9/17 (53%)	11/48 (23%)	9/39 (23%)
3	3/17 (18%)	13/48 (27%)	11/39 (28%)

Age is displayed in median (IQR). All other variables are displayed in n/group total (%). All patients had CURB-65 1, 2 or 3.

Table 4.2 Detection of pathogens per aetiological group

Pneumococcal CAP (n=48)	***Sputum culture****	***Blood culture***	***Urinary antigen test***
S. pneumoniae	13/25 (52%)	18/48 (38%)	30/48 (63%)

Strict viral pathogens (n=17)	**Oropharyngeal swab**
Influenza A/B virus	7/17 (41%)
RS virus	4/17 (24%)
Para-influenza virus	3/17 (18%)
Coronavirus	2/17 (12%)
Human metapneumovirus	1/17 (6%)
Bocavirus	0/17 (0%)

Mixed pathogens (n=39)**	**Influenza**	**RSV**	**PI**	**CV**	**hMPV**
S. pneumoniae	9/39 (23%)	3/39 (8%)	2/39 (5%)	-	3/39 (8%)
H. parainfluenzae	6/39 (15%)	-	1/39 (3%)	-	3/39 (8%)
H. influenzae	4/39 (10%)	1/39 (3%)	-	2/39 (5%)	-
M. catarrhalis	3/39 (8%)	-	-	1/39 (3%)	1/39 (3%)
S. aureus	1/39 (3%)	1/39 (3%)	1/39 (3%)	-	1/39 (3%)
E. coli	1/39 (3%)	1/39 (3%)	1/39 (3%)	-	-
K. pneumoniae	-	-	-	-	1/39 (3%)
K. oxytoca	-	-	-	-	1/39 (3%)
S. marcescens	1/39 (3%)	-	-	-	-

Relative number (percentage) of patients in which the microorganism was detected per ae-tiological group. Influenza, influenza virus A or B; RSV, respiratory syncytial virus; PI, parainfluenza virus; CV, corona virus; hMPV, human metapneumovirus. aln the pneumococcal group, for (48 - 25=) 23 patients no sputum sample was obtained. bln the mixed aetiology group, for (39 - 32=) 7 patients no sputum sample was obtained.

Table 4.3 Levels of plasma cytokines in the three CAP groups

	Aetiology				
***Cytokine** (pg/mL)*	**Strict viral**	**Pneumococcal**	**Mixed**	**P-value**	**LLOD**
	n=17	**n=48**	**n=39**		
IL-1β	1.6 (2.12)	1.6 (1.95)	1.6 (1.56)	0.960	0.167
IL-4	0.00 (2.38)	0.00 (0.47)	0.00 (0.47)	0.591	0.639
IL-5	2.21 (3.95)	1.22 (2.04)	1.22 (1.51)	0.401	0.352
IL-6	12.9 (55.5)	378 (1444)	66.6 (288)	<0.001	2.03
IL-10	5.3 (10.8)	12.1 (30.4)	6.78 (28.5)	0.048	0.090
IL-12p70	0.00 (0.00)	0.00 (0.10)	0.00 (0.04)	0.135	0.397
IL-17A	0.00 (0.00)	0.04 (0.25)	0.00 (0.04)	0.002	0.101
IL-23	0.00 (0.13)	0.00 (0.26)	0.00 (0.26)	0.207	0.733
IL-27	0.00 (10.7)	0.00 (17.6)	0.00 (28.3)	0.248	5.35
IFN-γ	4.88 (12.3)	0.97 (1.70)	1.45 (1.45)	0.002	0.305
TNF-α	0.00 (0.56)	0.00 (0.29)	0.03 (0.50)	0.531	0.249
IP-10	91.8 (210)	78.5 (142)	116 (233)	0.484	0.560

Median (IQR) cytokine concentrations per group in picogram/ml. For IL-17A, median plasma levels are just below Lower limit of detection (LLOD) of the Luminex assay, but reliably extrapolated from the calibration curve in 19 subjects. IL-21, IL-22 and TNF-α are not displayed as they were undetectable in all samples.

Table 4.4 Two created cytokine prediction models, with and without CRP

Cytokine prediction model			
	B	**OR**	**95% confidence interval**
IL-5 (pg/mL)	0.076	1.079	0.988 – 1.178
IL-6 (pg/mL)	-0.008	0.992	0.986 – 0.999
IFN-γ (pg/mL)	0.053	1.055	0.992 – 1.121
Constant	-1.156	0.315	

Cytokine and CRP prediction model			
	B	**OR**	**95% confidence interval**
IL-6 (pg/mL)	-0.008	0.992	0.986 – 0.999
IL-27 (pg/mL)	-0.050	0.951	0.907 – 0.997
CRP (mg/L)	-0.018	0.982	0.971 – 0.993
Constant	1.892	6.629	

Top panel of the table: cytokine-based prediction model. Bottom panel: cytokine plus CRP-based prediction model. Odds ratio's in these models are theodds of having strict viral CAP compared to the odds of having pneumococcal or mixed CAP, for every unit change in plasma level of that cytokine. B, regression co-efficient per unit change in plasma level; OR, Odds ratio per unit change in plasma level; 95% confidence interval for given odds ratio's.

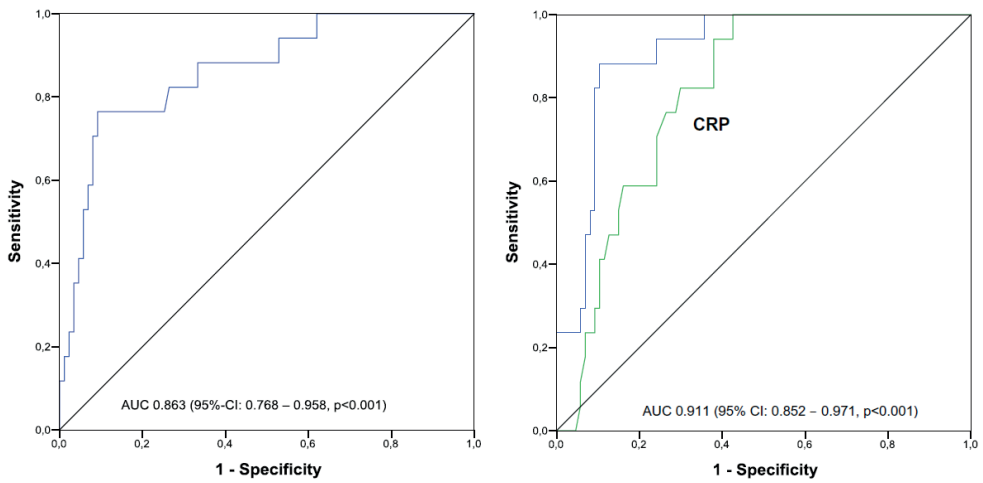
When adding CRP in the selection method, the cytokines IL-6, IL-27 and CRP appeared to distinguish viral from other aetiologies of CAP. In this model, 59% of the variance in subjects either having either strict viral or the other two aetiologies of CAP (mixed and pneumococcal) can be explained by the cytokines IL-6, IL-27 and CRP (R2 = .587). Figure 4.2 shows that the ROC curve of the predicted probabilities from all subjects with viral CAP, had an AUC of 0.911 (95% CI: 0.852-0.971, $P < .001$). Using a cut-off value of 0.65, a specificity of 99% and sensitivity of 35% was found. In comparison, prediction by CRP alone would have an AUC of 0.813 (95% CI 0.728-0.898, $P < .001$).

When doing a sensitivity analysis on all viral CAP patients without using PCT as a selection criterion ($n = 27$), we created two extra ROC curves with lower AUC values.
A cytokine-only prediction model had an AUC of 0.726 (CI:0.624-0.829, P < .001). The prediction model using both cytokines and CRP has an AUC of 0.775 (CI: 0.681-0.870, P < .001).

DISCUSSION

In the present study, systemic cytokine levels differed between patients classified upon documented microbiological cause of CAP. Differences were observed for IL-6, IL-10, IL-17A and IFN-γ, yet with considerable overlap for all cytokines between all groups. Cytokine measurements may add predictive value to the routine clinical measurements of CRP. However, current findings cannot be extrapolated to an unselected cohort of patients, thereby being merely a proof of concept. These findings need prospective validation in unselected patients to determine predictive values and the effects on patient management and patient outcome.

Figure 4.2 ROC curves of both multiple cytokine prediction models for distinction between strict viral and the combined pneumococcal and mixed aetiology group, using two multiple cytokine prediction models



The left picture displays the first (cytokine-alone) model. The right figure displays the second (cytokines + CRP) model in blue (AUC 0.911 (95% CI: 0.852-0.971, P < .001) and prediction by CRP alone in green as a reference (AUC of 0.813 (95% CI 0.728-0.898, P < .001).

Previous studies have focused on individual biomarkers to differentiate aetiology in CAP. The best-studied biomarker is the plasma IL-6 level. IL-6 proved to differentiate between typical and viral CAP, between pneumococcal CAP and *Mycoplasma pneumoniae* and between pneumococcal and non-pneumococcal CAP respectively[26-28]. Interestingly, CAP severity scores and mortality have been found to correlate well with cytokine IL-6 levels on the first day of hospitalization[29,30]. This poses the question whether cytokine levels are specific for aetiology or specific for disease severity, or both. Menendez et al[7]. demonstrated that peripheral IL-6 is elevated in CAP presenting with acute sepsis or shock despite the aetiology, but is also elevated in CAP caused by Gram-positive cocci without septic shock. Endeman et al[26]. reported that IL-6 levels in blood predict pneumococcal CAP when independent of age and PSI. In our analysis, CURB-65 score alone or added to our multivariable models did not improve prediction of aetiology (data not shown). Unlike other studies, patients directly admitted to the ICU were excluded in the REDUCE study, consequently data of these more severely ill patients are not available.

The major limitation of the present study is that the analyses were retrospective (although the data and samples were prospectively collected), with extremely well-selected and defined patient groups and with exclusion of patients in which no pathogen was detected. We used a PCT cut-off of <0.25 µg/L to select a group in which it was highly unlikely that a bacterial co-infection was present. We believe this is necessary as a criterion to exclude possibly bacterial and viral co-infections, since the microbiological techniques used are not sensitive and specific enough to reduce this possible bias. Prospective validation in an unselected cohort is needed to validate our results and compare them with other diagnostic strategies to withhold or withdraw antibiotics, such as solely PCT or CRP. In a sensitivity analysis, we saw CRP alone predicted less than CRP and cytokines combined. This suggests cytokines add value to the prediction of viral CAP. In a second sensitivity analysis, we excluded the use of PCT as a selection criterion. The then-created ROC curves for the correlation predicted worse, with lower AUC values. Thus, we believe PCT may be needed as a selection criterion in order to exclude undetected bacterial pathogens from the viral group, which creates a model predicting better than without the PCT criterion.

Choosing a PCT criterion lowers, but not completely rules out, the chance of including individuals with undetected bacterial pathogens to our strict viral CAP group[31]. Low PCT was found to differentiate typical from atypical CAP, but not atypical from viral CAP[14,32]. So, atypical pathogens that were not detected by PCR, could potentially have been selected in our presumably strict viral group.

Exclusion of patients with an indefinite microbial cause may create considerable bias. Menendez et al.[7] reported higher l-6 levels in the known-aetiology group compared to the unknown-aetiology group. Only patients with a definite microbial diagnosis were selected for our model, and in general a definite microbiological diagnosis is established in only 40%-50% of patients presenting with CAP[4-7].

Furthermore, group definitions may influence results. We choose to exclude adenoviruses and rhinoviruses because of the uncertainty to assign them to be the causative pathogen in patients in which this is the only pathogen found. We choose the pneumococcal CAP group for its homogeneity, but did not select other bacterial CAP groups, because these groups would be too small for meaningful statistical analysis. We expected the mixed CAP group to be heterogeneous, both in aetiology as in cytokine expression. Subgroup analysis was not reliable with a maximum of nine subjects having comparable co-infection (influenza-pneumococci). Noteworthy, is the absence of atypical pathogens in our mixed CAP group, despite the use of routine PCR on oropharyngeal swab, which increases detection rate[33].

We did not take pretreatment with antibiotics or prednisone prior to admission into account. Previous studies stated that antibiotic pretreated patients had lower IL-10 and IL-6 levels, compared to treatment-naive patients[7,28]. Endeman et al.[26] reported that patients treated with corticosteroids on admission or prior to admission had significantly lower IL-6 levels on day 3 compared to patients not treated with corticosteroids at all. Furthermore, we are unsure about the effect of COPD on our results, since chronic lung inflammation in COPD patients may alter the immune response towards a Th-1 direction[34]. The pitfall of CAP in COPD patients is that causality between pathogen detection and disease is challenging. For example, 25% of ambulant COPD patients carry clinically significant amounts of pathogens in the lower airways while not suffering from an exacerbation, compared to 52% potential pathogens during exacerbation[35].

Timing of measurement may have influenced the results as well. Cytokine levels generally decline in the course of disease, influence other pathways or alter after treatment is initiated[26,36]. These factors should be taken into account in future research aimed at further validating the prediction model. Technical aspects might have influenced our results as well. TNF-alpha, for example, was low or even undetectable. Collection of samples was highly standardized (rapid processing after blood withdrawal) and analysis were performed by experienced personnel in a highly standardized setting and appropriate controls were used. Some technical variation in detection, for example, by the use of different Luminex panels, might have also influenced our TNF-alpha results.

Rapid cytokine tests such as Simple Plex are found to be as accurate as Luminex, but require less time and human effort[37]. Future research is needed to investigate the benefit and pitfalls of a rapid, cytokine-based prediction model and if this predicted diagnosis is reliable enough to base treatment strategy on. When validating this studies' prediction model prospectively, up to half of all patients will likely not have a definite microbial diagnosis. Before implementing an eventual strategy to withhold antibiotics based on this model, it should be validated in prospective trials and a costeffectiveness analysis should be performed. Several outcome variables such as length of stay, treatment failure and mortality should be documented. Finally, costs of cytokine assays and analysts performing it should outweigh the consequences of antibiotic resistance, for this approach to be cost-efficient.

In the present retrospective cohort analysis, we found pathogen specific differences in cytokine levels of CAP patients on admission. Combination of predictive value of cytokines seem promising for validation in prospective research, especially concerning viral aetiology. Future research should focus on validating our findings in a prospective unselected cohort of patients with CAP.

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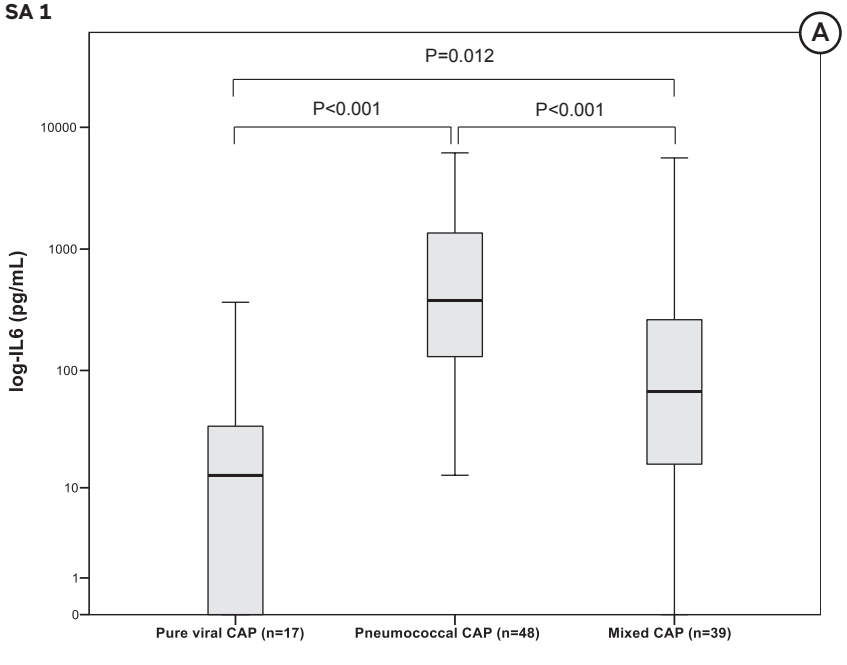
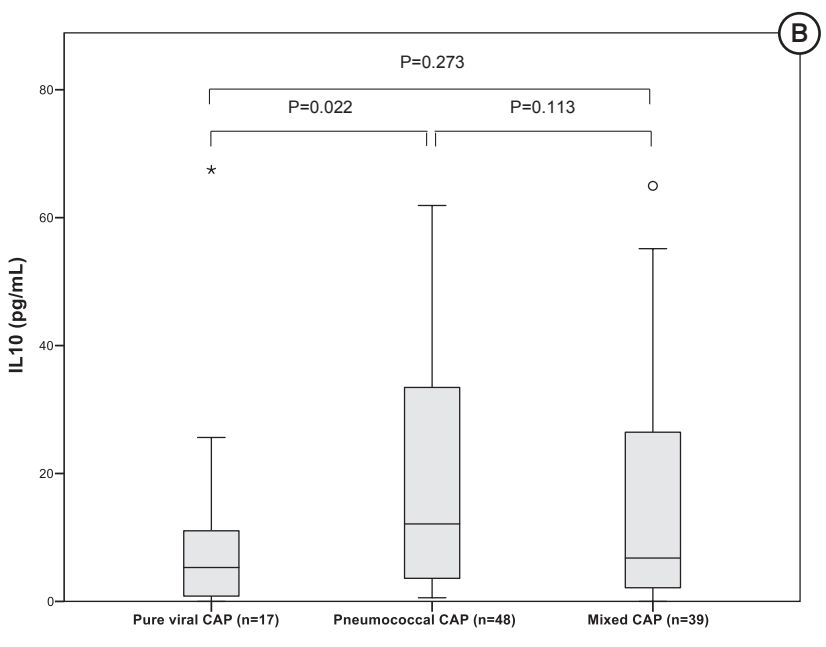
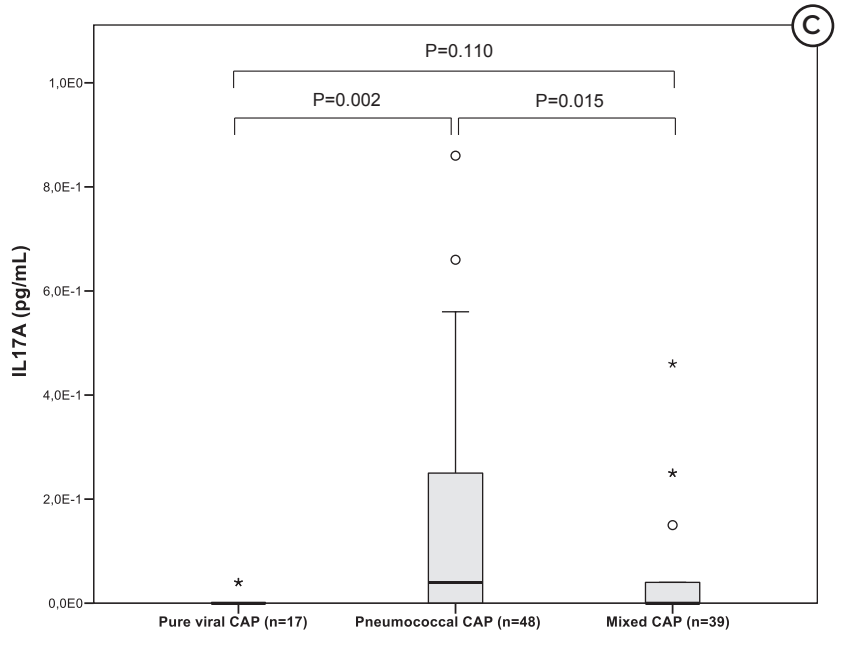
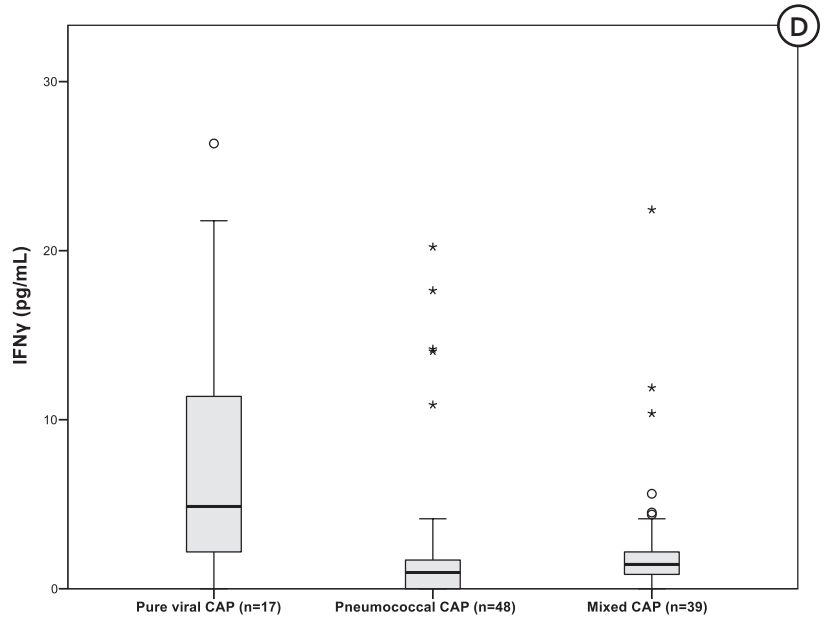
Appendices

Figure SA 1



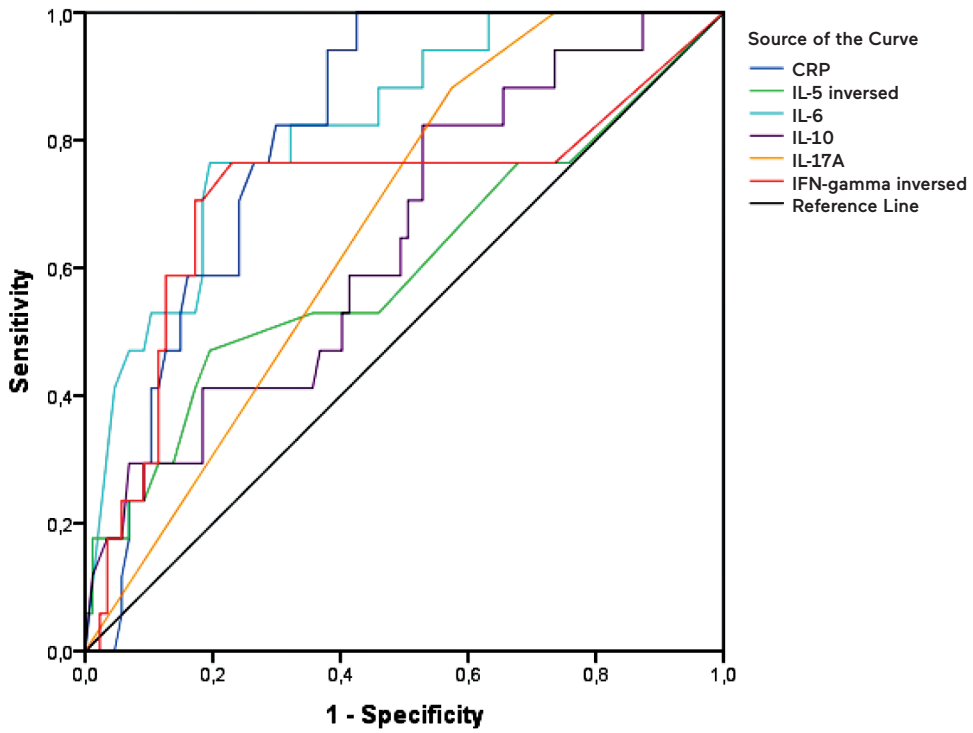






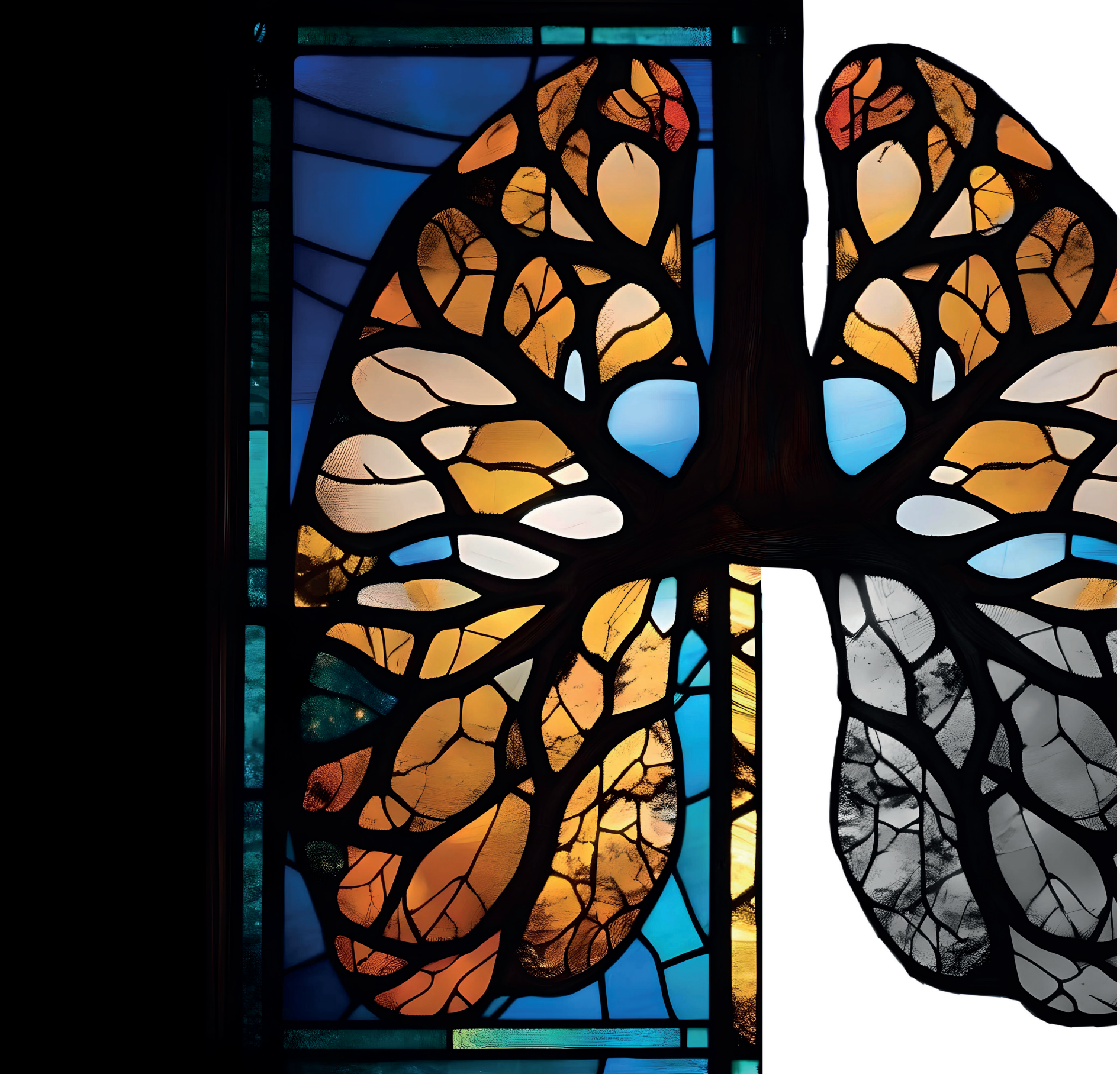
Distribution of cytokine plasma levels per aetiologic group. **A:** For IL-6, the strict viral CAP group displays a reasonable overlap with the lower quartile of the pneumococcal group and the mean of the mixed group. **B:** Plasma IL-10 levels only differ significantly between strict viral and pneumococcal CAP. **C:** IL-17A levels in the strict viral CAP group was out of range for the Luminex detection in all but one of group subjects. **D:** for IFN-γ both pneumococcal and mixed CAP comprises relevant outliers for comparison with strict viral CAP.

Figure SA2



ROC curve to distinguish strict viral from the non-strict viral group of all individual cytokines used in the prediction model: IL-6, IL-10, IL-17A, IFN-γ (inversed) and IL-5 (inversed). IFN-γ and IL-5 are inverted for interpretation purposes, because these cytokines are elevated in strict viral CAP, unlike the other cytokines which are elevated in pneumococcal or mixed CAP. Diagonal segments are produced by ties.
CRP: AUC 0.813 (95%-CI: 0.728 – 0.898, p<0.001); IL-6 AUC 0.824 (95%-CI: 0.722 – 0.926, p<0.001); IFN-γ AUC 0.714 (95%-CI: 0.551 – 0.877, p=0.005); IL-17A AUC 0.669 (95%-CI: 0.552 – 0.786, p=0.028); IL-10 AUC 0.645 (95%-CI: 0.505 – 0.785, p=0.059); IL-5 AUC 0.602 (95%-CI: 0.436 – 0.769, p=0.183); IL-10 and IL-5 had a non-significant area under the curve when used as a single predictor for viral CAP.

Appendices



Chapter 5

Biomarker guided antibiotic stewardship in Community Acquired Pneumonia: a randomized controlled trial

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Accepted for publication PLoS One

ABSTRACT

Background

In community-acquired pneumonia (CAP), the role of biomarkers to shorten duration of antibiotic treatment has not been firmly established.
We assessed the effectiveness of active feedback of treatment algorithms based on procalcitonin (PCT) and C-reactive protein (CRP), compared to standard care, on the duration of antibiotic treatment in patients hospitalized with community-acquired pneumonia (CAP) in non-ICU wards.

Methods and findings

We performed a randomised, open label, parallel group, multi-centre trial in 3 Dutch teaching hospitals. Treatment was guided by a PCT algorithm, CRP algorithm or standard care. Participants were recruited by a member of the study team and randomised at day 2-3 of admission in a 1:1:1 ratio. Treatment was discontinued upon predefined thresholds of biomarkers that were assessed on admission, day 4 and days 5-7 if indicated. The primary outcome was total days on antibiotic treatment until day 30.
In total 468 participants were included in this study. The median days on antibiotics (IQR) was 7 (IQR 7-10) in the control group, 4 (IQR 3-7) in the CRP group (rate ratio (RR) of 0.70, 95% CI 0.61 - 0.82 compared to standard care; p <0.001), and 5.5 (IQR 3-9) in the PCT group (RR of 0.78, 95% CI 0.68 - 0.89 compared to standard care; p <0.001). New antibiotics within the first 30 days were prescribed to 24, 23 and 35 patients in standard care, CRP and PCT groups, respectively. The hazard ratio for a new prescription in patients in the PCT group compared to standard care 1.63 (CI 0.97 - 2.75; p = 0.06). No difference in time to clinical stability or length of stay was found.

Conclusion

A strategy of feedback of CRP-guided and PCT-guided treatment algorithms reduced the number of days on antibiotic in the first 30 days after hospital admission in non-ICU wards for CAP. The study was not powered to determine safety of shortening duration of antibiotic treatment. (NCT01964495)

INTRODUCTION

Community-acquired pneumonia (CAP) is an important cause of death worldwide[1]. In Europe 3.3 million people develop CAP per year, of whom 20-50% need hospital admission. The annual costs associated with CAP in Europe amount to ~€10.1 billion, with inpatient care accounting for €5.7 billion and treatment accounting for ~€0.2 billion[2,3].

Guidelines recommend antibiotic courses of five to 21 days, depending on severity of illness, causative pathogen, clinical response and type of antibiotic used[4-6]. Yet, in daily practice physicians tend to treat longer than recommended, especially in patients with significant comorbidities, in patients who fail to respond rapidly on antibiotic treatment and in patients with severe CAP[7-10].
This underlines the need for guidance to shorten the duration of antibiotic treatment without compromising patient safety.
Biomarkers have been proposed as objective means to tailor antibiotic treatment in patients with CAP. PCT is the most studied biomarker, which seems useful to withhold or discontinue antibiotics in patients with acute respiratory infections, including CAP, without an increase in treatment failure or mortality[11,12]. However, concerns have been raised regarding patient selection in clinical trials, non-adherence to PCT algorithms by treating physicians and usefulness of PCT in patients with atypical pathogens or renal failure[13,14].
In the Netherlands C-reactive protein (CRP) is the most commonly used biomarker in patients hospitalized with CAP. Results from two observational studies in patients with CAP suggested that CRP might aid the clinical decision-making process[15,16].
We, therefore, performed a randomised controlled multi-centre trial to quantify the effects of a CRP and a PCT based algorithm, compared to routine care, on the duration of antibiotic treatment duration in hospitalized patients with CAP.

METHODS

Trial design and oversight

This is a multi-centre randomised controlled parallel group open-label trial involving patients hospitalized with CAP in non-ICU hospital wards of three teaching hospitals in the Netherlands (The Northwest hospital Alkmaar, ISALA clinics Zwolle and the Slotervaart Hospital in Amsterdam).

Prior to the trial, all participating centres were familiar with CRP measurements in routine care. None of the participating centres used PCT in routine care.
The study protocol was approved by the Medical Ethics Committee associated with the Northwest hospital (METC- registration: M013-031, CCMO-registration NL44806.094.13) and is in full compliance with the Helsinki declaration. The study protocol was registered in the clinicaltrials.gov database. (NCT01964495)
Eligible patients were approached for written informed consent twice. At the time of

admission a short written informed consent was obtained to collect a blood sample for determination of PCT levels, as this was not part of standard care. At day two or three prior to randomisation a full written consent was obtained.
Recruitment started December 5, 2013 in the Northwest hospital, followed by the Slotervaart Hospital in July 2014 and finally the ISALA clinics in March 2015. Recruitment was completed in all hospitals in October 28, 2016. The authors vouch for the quality of the data collection and analysis.

Participants

All adult patients with a clinical diagnosis of CAP made by the attending physician were assessed for eligibility. The attending physician made the decision whether or not the patient required hospitalization or ICU admission based on routine care. Patients with radiologically confirmed CAP admitted to a non-ICU ward without severe immunosuppression, active neoplastic disease, obstruction pneumonia, or aspiration pneumonia, were eligible for the study (see supplementary material S5.1 for full criteria). If another diagnosis was established prior to randomisation and antibiotic treatment was stopped, patients were not randomised and excluded from analysis (Figure 5.1).

Simple size calculation

Sample size calculation was done using the program G-power. We assumed a mean (SD) treatment duration of 8.8 (SD ±5.9) days based on routine clinical practice in the Northwest clinics and hypothesized that treatment duration could be shortened by 2 days by using the CRP and PCT-based algorithms. Using an α of 0.025 (corrected for multiple testing using the Bonferroni Holm method) and β of 0.20 resulted in a total of 139 patients required per group assuming a normal distribution of the primary endpoint and of 146 patients per group with non-normal distribution. We included a total number of 156 patients per group to account for loss to follow-up, deaths etc. which amounts to a total number of 468 patients.

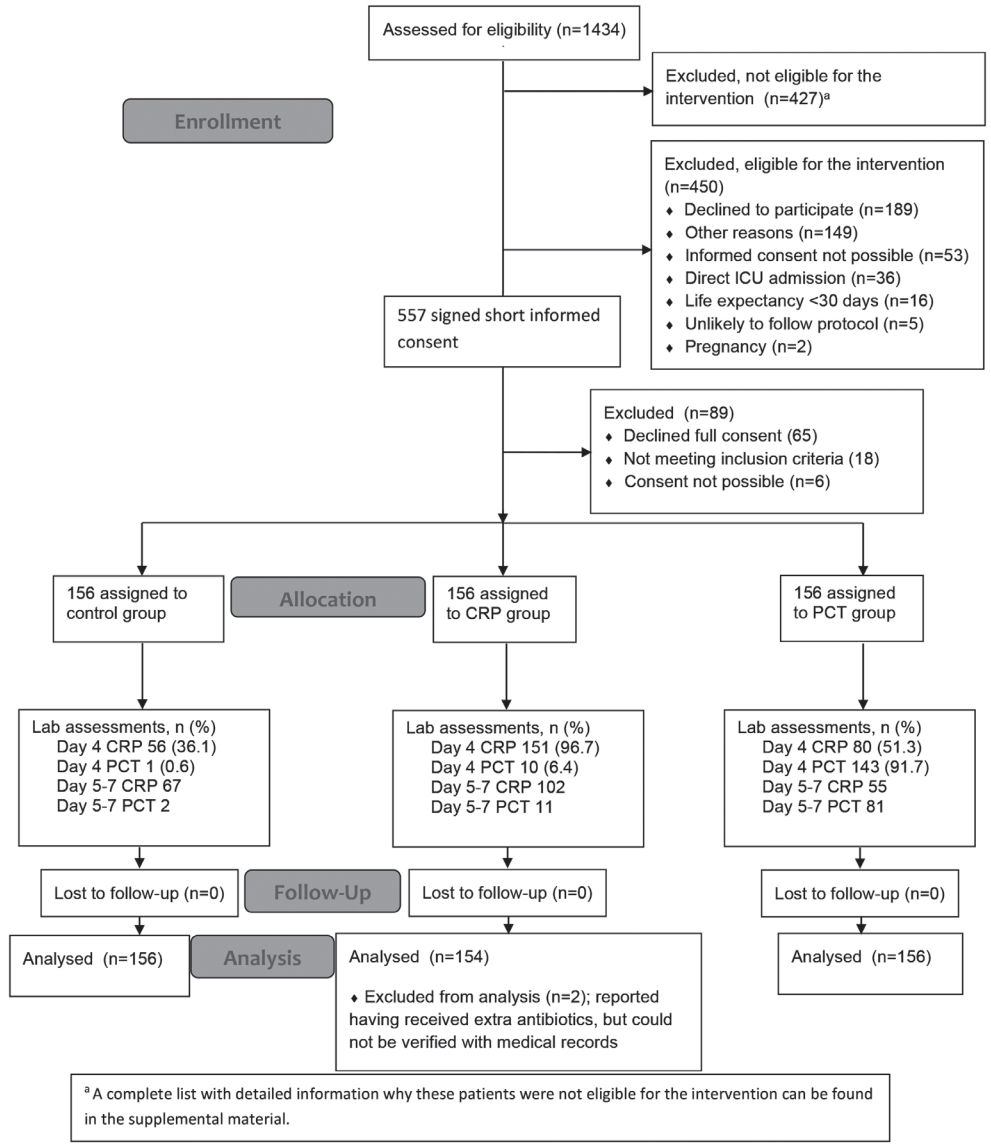
Randomisation

We compared patients treated according to current guidelines (control group) with patients in whom antibiotic treatment was guided by serum PCT levels (PCT group) or by serum CRP levels (CRP group). Randomisation was performed on day two or three of admission in a 1:1:1 ratio by means of block randomisation using blocks of 30 patients at a time and one final block of 18. Blocks were generated by an independent statistician. Each centre was assigned a block and upon completion was assigned the next block of 30. Patients were allocated to one of the three groups by sealed opaque envelopes. After randomisation no masking was performed.

Interventions

Baseline assessment included clinical data, vitals, comorbid conditions, medication and routine blood tests. Chest x-rays were reviewed by attending physicians, who also decided whether or not to start empiric CAP treatment.

Figure 5.1 CONSORT 2010 Flow Diagram



Standard microbiological tests consisted of blood cultures, sputum culture (if possible), urinary antigen tests for pneumococci and legionella and an oropharyngeal swab for multiplex PCR for atypical and viral pathogens (RespiFinder® 2SMART version 2.2 en 2.3). All results were reported to treating physicians according to routine practice.
Patients were treated according to Dutch national guidelines for the first three days of admission[6]. The first day of admission was defined as day one, even if a patient was admitted in the evening and only received one dose of antibiotics. Consequently, if antibiotics were stopped on day four, duration of treatment was counted as three days. In the control group the duration of antibiotic therapy was based on national guidelines and the time of stopping antibiotics was left to the discretion of the attending physician. In all study groups, physicians were free to order routinely available diagnostic tests, during the patients' hospital stay.

CRP analysis was performed using C-reactive protein reagent and the Beckman Synchron DxC 800 analyzer (Beckman Coulter Inc., Brea, California, USA). Serum samples were analyzed within 2 hours after collection. PCT analysis was performed using the Vidas B.R.A.H.M.S. PCT assay and the Vidas immunoanalyzer (BioMerieux, Marcy l'Etoile, France). Serum samples were analyzed within 2 hours after collection.

CRP and PCT were determined on day 1 for all patients and then in the intervention groups on day four. If the day-4 level was below the threshold value (below 100 mg/L and a reduction to below 50% of the initial value for CRP and below 0.25 µg/L or a reduction to below 10% of the initial value for PCT) antibiotics were discontinued. If the level was not low enough to discontinue antibiotics, CRP or PCT was determined daily until the threshold was reached until day 7 at the latest. If patients were discharged before antibiotics were stopped, additional blood samples were collected during outpatient visits or home visits. These patients were informed about continuing or stopping antibiotics by a member of the research staff, who are all physicians of the pulmonology department. In case of any doubt whether or not antibiotics could safely be discontinued when the patient was still exhibiting symptoms, this decision was left up to a senior member of the pulmonology staff.
All outcomes were assessed at an outpatient visit at day 30±2.
CRP threshold values were derived from the CAPISCE study, a clinical trial in patients with CAP with daily measurement of biomarkers during the first week of admission[17].
PCT threshold values were derived from the study performed by Christ-Cain et al[18].
Attending physicians regularly received in-person training on study protocol and were allowed to deviate from protocol for safety reasons. Reasons for protocol deviations were documented. All biomarker results were actively checked by a member of the research staff and communicated to the treating physician.

Outcomes

The primary endpoint was total number of days on antibiotic treatment until day 30. This includes IV and oral treatment. Secondary endpoints consisted of new antibiotic prescriptions, length of stay, time to clinical stability and all-cause mortality, all with a time-window of 30±2 days from admission. All outcomes were assessed and recorded by a designated member of the research team in each centre.
New antibiotic prescriptions were defined as: any broadening, prolongation beyond originally planned treatment duration (including prolongation of treatment beyond the recommended duration as determined by the biomarker algorithms in the intervention arms), or restarting of antibiotic treatment during the intervention period (day four and onward). Reasons for new prescriptions were documented. Only the first new prescription was counted as an event. Originally in our protocol the term treatment failure was used instead of new antibiotic prescriptions, but the latter is more accurate so is used throughout this manuscript instead. The definition of this term remains unchanged.
Clinical stability was defined according to the criteria mentioned in the IDSA/ATS CAP guideline[5]. This list can be found in the supplementary material.

Statistical analysis

All data was analysed using IBM SPSS statistics version 20 for Windows and R statistics. Initially we planned to analyse the primary endpoint by comparing means/medians with standard parametric or non-parametric tests, however upon completion of our trial we realized that a negative binomial model with robust standard errors would be more appropriate as it yields an effect size rather than a p-value, so we changed our analysis accordingly. We used robust standard errors because due to our study design most patients are either treated for three days or seven days, which violates the distributional assumption of a negative-binomial model. The primary outcome is reported as Rate Ratios (RR) with 95% confidence intervals, reflecting the relative change in the number of calendar days on antibiotic treatment.
Length-of-stay was analysed using a Cox proportional hazards model and a competing events regression model with death as a competing variable using R statistics.
All other outcomes were assessed using a Cox proportional hazards model and reported with hazard ratios and 95% confidence intervals. Any patient that died during hospital admission was censored in the analysis for length of stay. Patients that did not meet criteria for clinical stability prior to discharge or died during hospital admission were censored in the analysis for time to clinical stability.
Patients were analysed according to the allocated intervention, i.e. using an intention-to-treat approach.

RESULTS

Participants

1434 patients were screened for eligibility and 895 met the in- and exclusion criteria, of which 557 signed the short informed consent for assessing PCT values at the time of admission. Of these, 468 were randomised, as 65 declined full informed consent, 18 did not meet in- and exclusion criteria on admission, four patients could not consent due to delirium and inability to reach a legal representative and in two patients palliative care was started prior to informed consent. Reasons for non-inclusion and non-randomisation are detailed in figure 5.1. Baseline characteristics are outlined in table 5.1. Results of all microbiological tests appear in supplementary table SE1. In 297 (63.5%)

Table 5.1 Baseline characteristics on admission[a]

	Control group (n=156)	CRP group (n=156)	PCT group (n=156)
Age, yr.	67 ± 16	68 ± 15	67 ± 15
Male sex, no. (%)	94 (60.3)	92 (59.0)	87 (55.8)
Smoking status			
Former smoker, no. (%)	76 (52.8)	84 (58.3)	81 (54.4)
Current smoker, no. (%)	42 (29.2)	33 (22.9)	37 (24.8)
Pack years of current and former smokers, median (IQR)	30 (16-42.3)	32 (13-48)	30 (16.5-50)
Antibiotic pre-treatment, no. (%)	39 (25.3)	38 (24.7)	31 (20.4)
Coexisting illnesses, no. (%)			
Congestive heart failure	20 (12.8)	21 (13.5)	19 (12.3)
Cerebrovascular disease	8 (5.1%)	16 (10.3%)	9 (5.8%)
Chronic renal disease	9 (5.8%)	5 (3.2%)	9 (5.8%)
Liver disease	1 (0.6%)	1 (0.6%)	2 (1.3%)
Diabetes Mellitus	16 (10.3)	21 (13.5)	22 (14.1)
COPD	46 (32.2)	51 (35.4)	53 (35.6)
Charlson comorbidity index, median (IQR)	1.00 (0-2)	1.00 (0-2)	1.00 (0-2)
Examination			
Body temperature, °C	38.3 ± 1.0	38.2 ± 1.1	38.3 ± 1.0
Oxygen saturation, %	93.9 ± 3.6	93.6 ± 4.4	93.2 ± 4.0
Supplemental Oxygen, no (%)	104 (66.7)	106 (67.9)	105 (67.3)
Heart rate, beats/min	97.5 ± 19.8	99.7 ± 20.5	101.0 ± 20.2
Systolic blood pressure, mm Hg	131.6 ± 21.6	132.2 ± 22.6	132.1 ± 22.2
Diastolic blood pressure, mm Hg	74.0 ± 14.4	74.6 ± 13.7	75.0 ± 15.6
Laboratory findings on day 1			
Procalcitonin (µg/L), median (IQR)	0.475 (0.120-4.053)	0.510 (0.120-5.100)	0.555 (0.113-4.825)
C-reactive protein (mg/L), median (IQR)	182 (96-249)	162 (80.5-265)	154 (85-257)
Leukocyte count (x 10^9/L), median (IQR)	14.05 (11.23-17.45)	12.6 (9.70-17.58)	13.8 (10.20-18.10)
PCT < 0.25 µg/L, no. (%)	59 (37.8)	59 (37.8)	64 (41.0)
CRP ≥ 100	114 (73.1)	110 (70.5)	109 (69.9)
CRP 50-100 mg/L, no. (%)	17 (14.9)	26 (16.7)	28 (17.9)
CRP < 50 mg/L, no. (%)	25 (16.0)	20 (12.8)	19 (12.2)
Imaging, no. (%)			
Pleural effusion	33 (21.2)	24 (15.5)	29 (18.6)
Multilobar pneumonia	44 (28.2)	47 (30.1)	44 (28.2)
CURB-65 score, median (IQR)	1 (0-2)	1 (1-2)	1 (1-2)
CURB-65 score ≥3 no. (%)	21 (13.5)	24 (15.4)	22 (14.1)
Empiric regime covering atypical pathogens, no (%)	48 (31)	61 (39)	37 (24)

a Plus-minus values represent means ± SD. The conversion factor for procalcitonin is: µg/L * 0.161 = nmol/L.

patients a potential pathogen could be identified. An overview of co-infections appears in supplementary table SE2.

Intervention

On admission CRP and PCT were determined in 468 and 464 patients, respectively. At day four, CRP was determined in 56 (36.1%) in the control group, in 151 (96.7%) in the CRP group and in 40 (25.6%) in the PCT group. At day four, PCT was determined in 1 (0.6%) in the control group, in 10 (6.4%) in the CRP group and in 143 (91.7%) in the PCT group. Follow-up CRP measurements after day four were performed in 67, 102 and 55 patients in the control, CRP and PCT group, respectively. Follow-up PCT measures after day four were performed in 2, 11 and 81 patients in the control, CRP and PCT group, respectively. All patients complied with sample collection. In case of logistical errors, e.g. when samples for biomarker testing were not ordered , or samples were lost during transportation or processing, an attempt was made to collect another sample in time. If that failed, antibiotics were continued and a sample was taken the next day, up until day 7.

Primary outcome

Overall antibiotic use was reduced in both intervention groups, respectively 30% in the CRP group (median 4 vs. 7d; p <0.001) and by 22% (median 5.5 vs. 7d; p<0.001) in the PCT group. (Table 5.2) The rate of patients on antibiotic treatment during admission and follow-up is shown in figure 5.2. Primary outcome data were incomplete for two patients in the CRP group, because full information of antibiotic use up to day 30 was missing. Both patients were, therefore, excluded from analysis. Sensitivity analyses (assuming both patients had either not or both had received additional antibiotics for seven days) yielded similar interpretation. All patients complied with the study algorithm and stopped antibiotics when so instructed. In a post-hoc analysis the rate ratio of receiving antibiotics during the first 30 days was 1.11 (95% CI 0.93 - 1.32;, p = 0.129) for patients in the PCT compared to those in the CRP group.

Table 5.2 Primary outcome[a]

	Control group, n=156	CRP group, n=154[b]	PCT group, n=156
Days on antibiotic treatment			
Primary analysis			
Median (IQR)	7 (7 - 10)	4 (3 - 7)	5,5 (3 - 9)
RR (95% CI)	Reference	0.70 (0.61 - 0.82)	0.78 (0.68 - 0.89)
Sensitivity analysis 1: observed treatment days			
RR (95% CI)	Reference	0.73 (0.62 - 0.85)	0.78 (0.68 - 0.90)
Sensitivity analysis 2: observed treatment days +7			
RR (95% CI)	Reference	0.74 (0.63 - 0.86)	0.78 (0.68 - 0.90)

[a] In the originally planned analyses using non-parametric tests all p values comparing the CRP and PCT group to the control group were below p<0.001
[b] In the main analysis, two patients were excluded due to missing post-discharge antibiotic treatment data. In the sensitivity analyses all 156 patients were included

Figure 5.2

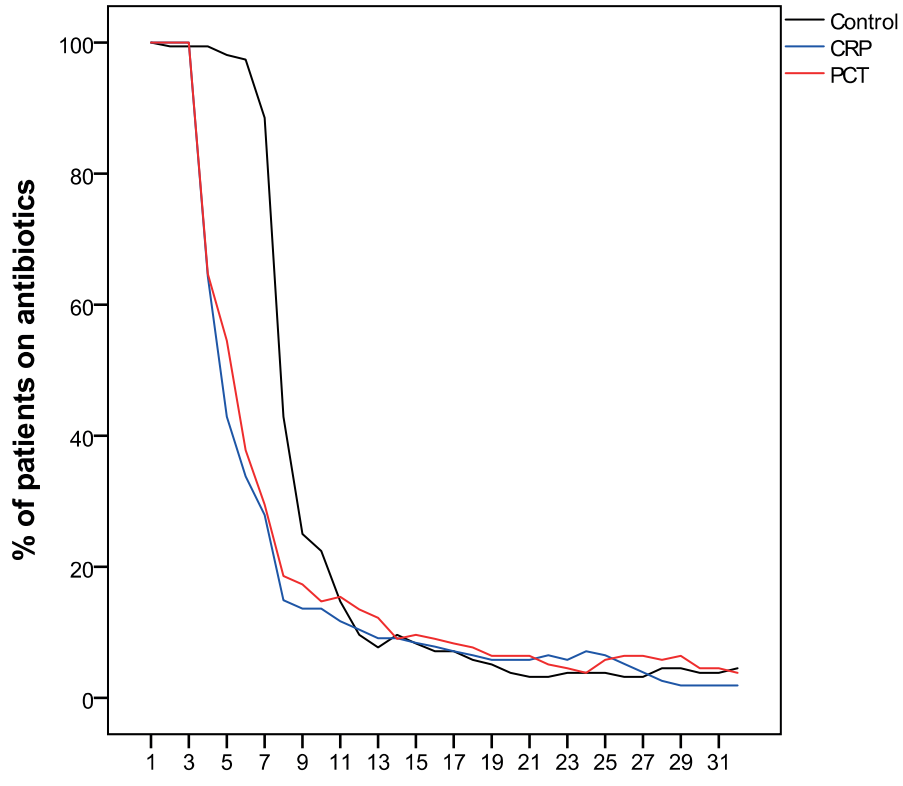


Table 5.3 Secondary outcomes

	Control group, n=156	CRP group, n=156	PCT group, n=156
New antibiotic prescriptions			
Intervention period day 4-30(±2)			
Number of cases (%)	24 (15.4)	23 (14.7)	35 (22.4)
HR (95% CI)	Reference	0.99 (0.56 - 1.76)	1.63 (0.97 - 2.75)
		p = 0.97	p = 0.064
Time to clinical stability[a]			
Median days (IQR)	3 (1-5)	2 (1-4)	3 (2-5)
Number of cases (%)	142 (91)	138 (88.5)	138 (88.5)
HR (95% CI)	Reference	1.07 (0.85 - 1.36)	0.85 (0.67 - 1.08)
		p = 0.55	p = 0.19
Length of stay			
Median days (IQR)	4.5 (3-7)	4 (3-6)	5 (3-8)
Cox regression HR (95% CI)	Reference	0.93 (0.74 - 1.17)	0.82 (0.66 - 1.03)
Competing events regression model[b]		0.98 ((0.80-1.19)	0.84 ((0.69 - 1.02)
30- day mortality			
Number of cases (%)	2 (1.3)	2 (1.3)	5 (3.2)

[a] In total 50/468 patients did not meet the criteria, 18 were in the CRP group, 18 in the PCT group and 14 in the control group. This was mainly due to either an elevated heart rate >100 bpm or low arterial oxygen saturation on room air that was due to known comorbidities (mainly COPD).
[b] Analysed using a competing risk regression model with death as a competing variable.

Secondary outcomes

All secondary outcomes are summarized in table 5.3. Additional antibiotics were prescribed in 82 (17.5%) patients. In 39 additional patients antibiotic treatment changed before day four and were not included in this analysis. A complete analysis including these patients appears in supplementary table SE3. In the intervention period a new antibiotic prescription was issued in 24 (15.4%), 23 (14.7%) and 35 (22.4%) patients in the control group, CRP group and PCT group respectively. The daily hazard ratios for new antibiotic prescriptions compared to standard care, were 0.99 (95% CI 0.56 - 1.76; p = 0.97) for the CRP group and 1.63 (95% CI 0.97 - 2.75; p = 0.064) for the PCT group. Reasons for new antibiotic prescriptions are listed in supplementary table SE4. Among those randomised to CRP-based treatment, a new course of antibiotics was started after discontinuation of the initial course based on the algorithm in 11 (7%) patients, and this occurred in 30 (19%) patients randomised to PCT-based treatment. Case summaries for all these patients can be found in the supplementary material.
9 patients (1.9%) had succumbed at day 30; 2 (1.3%) in the control group, 2 (1.3%) in the CRP group and 5 (3.2%) in the PCT group. Only 2 of these patients, both in the PCT group, received a shorter course of antibiotics according to study protocol. In both patients antibiotics were restarted due to relapsing fever. One patient died due to inhospital aspiration on the day she was set to be discharged. The other patient was treated for three days according to PCT levels, discharged at day four and readmitted three days after discharge with recurring pneumonia from which he recovered and was discharged. Two weeks after discharge he died from euthanasia due to end-stage COPD. In all other patients (n=5) that died before day 30 biomarkers remained high during antibiotic treatment, with the consequence that antibiotics could not be stopped before day seven.

DISCUSSION

In this randomised controlled trial both strategies of feedback of results from CRP-based and PCT-based algorithms for discontinuation of antibiotic treatment reduced antibiotic exposure during a 30 day follow-up period, compared to standard care in patients hospitalized with CAP in non-ICU wards.

Studies have shown that antibiotic duration can be shortened based on clinical parameters, such as the criteria for clinical stability defined in the IDSA guidelines[5,7,19]. However, some patients do not reach these criteria, even at the time of discharge.

Furthermore, despite all evidence that shorter antibiotic courses are safe, most physicians still treat patients hospitalized with CAP for 7-10 days[20, 21]. Although Dutch guidelines have recommended antibiotic courses of 5 days for patients with CAP and good clinical recovery by day 3 since 2011, 18 of 156 (11.5%) patients in the standard care treatment group were treated for less than 7 days in the current study. In another more recently performed Dutch multi-centre study addressing a similar patient population the average duration of antibiotic treatment was 6.6 days[10]. Antibiotic use is an important driver of antimicrobial resistance, and shortening of treatment duration reduces antibiotic selective pressure. This underlines the need for simple and objective criteria to change clinical practice.

In our study both CRP-based and PCT-based algorithms reduced the median days of antibiotic use in the first 30 days after admission from 7 to 4 and 5.5, respectively. A CRP based algorithm has advantages over a PCT based algorithm. CRP is a widely used, cheap(er) and readily available biomarker in nearly every clinical setting. Point-of-care CRP testing has proven effective in reducing antibiotic consumption for lower respiratory tract infections in nursing homes[22].

Yet, there is little evidence to support CRP measurements to tailor the duration of antibiotic treatment in patients with CAP. In one study failure of CRP to decline within the first few days of hospitalization was associated with a poor prognosis of CAP[23]. Only once has a CRP based algorithm been compared to a PCT based algorithm[24]. In that trial of 94 ICU-patients with sepsis, 49 were allocated to PCT and 45 to CRP measurements, without a control group. Median duration of treatment was 6 days in the CRP group and 7 days in the PCT group, with no differences in outcomes between groups. The same group studied a modified version of their CRP algorithm in open label RCT in ICU patients and found a small reduction in antibiotic treatment time in favour of the CRP group[25]. However, their algorithm differs from ours and a reduction of 50% was needed for patients with an initial CRP >100 mg/L and an absolute value of <35 mg/L was needed for patients with an initial value <100 mg/L.

Our PCT-based algorithm reduced antibiotic exposure but resulted in slightly more new antibiotic prescriptions during follow-up compared to the control group. This might have resulted, in part, from the fact that less patients in the PCT group received empirical therapy that covered atypical pathogens than the other study groups (table 5.1), which might have made them more prone to a change in antibiotic regime. Several other large, well-designed trials and a Cochrane review have been conducted in a variety of clinical settings but none of those reported more treatment failure in the PCT groups[11,12].
Christ-Crain et al. showed that in patients with radiologically proven CAP a PCT based algorithm reduced the duration of antibiotic treatment to a median of 5 days with similar rates of antibiotic prescription in long term follow-up. However, their algorithm was different from ours, they gave clinicians more freedom to take their own judgement into account and their definition of treatment failure was limited to symptoms related to CAP. Lastly combination therapy was started in 34% of patients, as compared to 24% of patients in our study.

Despite all evidence addressing PCT, there are still concerns about exclusion rates in clinical trials, uncertainty with regards to the necessity of overruling of the PCT algorithm in trials by treating physicians and the usefulness of PCT in patients with atypical pathogens, COVID-19, renal failure or critically ill patients[13,14,26-28].

Our study has several limitations. First, our study was underpowered to exclude harm due to reduced duration of antibiotic treatment, as recently recommended by the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) working group on design of antimicrobial stewardship evaluations[29]. Ideally such a trial would include all patients admitted with CAP and should demonstrate superiority in reducing antibiotic exposure over standard clinical practice and simultaneously demonstrate non-inferiority design for unwanted clinical outcomes. Short antibiotic courses carry the risk of undertreatment leading to recurrence or worsening of symptoms, additional antibiotic prescriptions, and increased time to recovery. Based on a meta-analysis of 26 RCT's there is no evidence that PCT based treatment strategies carry any of these risks[11]. In the current study 41 of 312 patients received a new antibiotic prescription after a short initial course based on the biomarker algorithms, and 23 of these prescriptions occurred within a week after antibiotic treatment was stopped. It is unlikely that all of these could have been prevented with a longer initial course, since some of these were due to culture results that returned resistant pathogens to empiric therapy.
The potential harm of short antibiotic treatment should be weighed against the harms of excess antibiotic treatment. Excess antibiotic treatment does not seem associated with lower rates of adverse outcomes, including death, readmission and emergency department visits[30].

Second, CRP measurements were frequently used in our control group and to a lesser extent in our PCT group. It is unclear if these measurements influenced clinical decision making and potentially study outcomes. If they did it would most likely lead to an underestimation of the effect of our CRP algorithm. In our PCT group CRP measurements did not influence antibiotic treatment duration. Even if they did, it would lead to an overestimation of the effect of the PCT algorithm.

Third, the observed 30-day mortality rate in our study is relatively low (1.9%), even though 15% of our patients classified as severe pneumonia according to the CURB-65 score. Reported 30-day mortality rates for hospitalised non-ICU patients with CAP range from 5-10%. The low mortality could have resulted from the study design, in which patients had to decide on day 2 or 3 on study participation, which may have selected for a less sicker study population. For instance informed consent on admission was not possible e.g. due to delirium in 53 patients, which could, therefore, not be included. Overall 450 out of 1434 screened patients were eligible for the intervention but were not included or randomized, compared to 468 randomized patients. This limits the generalisability of our findings. Fourth, post-discharge sampling to determine biomarkers was part of study protocol, but may not be realistic in routine daily care practices. In 62 of 88 patients in whom a blood sample was taken at home or on an outpatient visit, antibiotics were discontinued because of biomarker measurements. This also limits the generalisability of our results. Fifth, the feedback of biomarker results to treating

physicians was an important part of the intervention tested, implying that the effectiveness of the intervention may well be less when implemented without active feedback. Lastly, there is a considerable publication delay. However, the research question regarding biomarker based strategies is still relevant today, and the average treatment durations in our control group are comparable to those reported in similar patient populations in recently published studies[7,10].

In conclusion, in this study both CRP and PCT based treatment algorithms reduced the duration of antibiotic treatment in patients admitted to a regular hospital ward with CAP. Future studies should focus on the non-inferiority of this approach with respect to clinically relevant patient centered outcomes.

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Supplemental materials

eMethods 5.1 Inclusion and exclusion criteria

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Inclusion criteria

- Age ≥18
- Estimated life expectancy > 30 days
- Initial admission to a non-ICU ward
- A new infiltrate on chest radiograph
- Presence of one or more of the following signs and symptoms:
    - o Temperature ≥ 38^{0}C
    - o Dyspnoea
    - o Cough (with or without expectoration of sputum)
    - o Chest pain
    - o Malaise or fatigue
    - o Gastro-intestinal symptoms,
    - o Rales/rhonchi or wheezing,
    - o Egophony or bronchial breath sounds
    - o Haemoptysis.

Exclusion criteria

- Severe immunosuppression as judged by the investigator (e.g. HIV infection, chemo-therapy, immunosuppressive drugs with exclusion of low-dose corticosteroids)
- Active neoplastic disease
- Obstruction pneumonia
- Aspiration pneumonia
- Pneumonia that developed within eight days of hospital discharge
- Expected inability to comprehend or follow the study protocol
- Pregnancy
- Lactation
- Unable to give informed consent (either patient or legal representative)
- Suspected non-respiratory infection diagnosed prior to randomisation and requiring antibiotic treatment.

eMethods 5.2 Criteria for clinical stability

- Temperature ≤37.8°C
- Heart rate ≤100 beats/min
- Respiratory rate ≤24 breaths/min
- Systolic blood pressure ≥90 mm Hg
- Arterial oxygen saturation ≥90% or pO2 ≥60 mm Hg on room air
- Ability to maintain oral intake
- Normal mental status

eMethods 5.3 Antibiotic strategy according to Dutch Guidelines (SWAB 2011)

Severity	Antibiotic	Route	Dose	Freq.
Mild pneumonia				
1st choice	Amoxicillin	Oral	500-750 mg	q6h-q8h
2nd choice	Doxycycline	Oral	100 mg (first dose 200 mg)	q24h
Moderately severe pneumonia				
1st choice	Penicillin	IV	1 ME	q6h
	Amoxicillin	IV	1000 mg	q6h
Severe pneumonia				
Monotherapy	Moxifloxacin	IV / oral	400 mg	q24h
	Or			
	Levofloxacin	IV / oral	500 mg	q12h
Combination therapy	Penicillin	IV	1 ME	q6h
	ciprofloxacin	IV / oral	400 mg (po 500 mg)	q12h
Combination therapy	cefuroxime	IV	750-1500 mg	q8h
	or			
	ceftriaxone	IV	2000 mg	q24h
	or			
	cefotaxime	IV	1000 mg	q6h
	erythromycin	IV	500-1000 mg	q6h

eTable 5.1 Results of microbial testsa

	Sputum culture (n=297)	Blood culture (n=372)	UAT (pneumococci n=419, L. pneumophila n=415)	Oropharyngeal swab (PCR, n=436)	Any test n, (% of all participants)
S. pneumoniae	42	36	64		112 (23.9)
L. pneumophila	5		8		9 (1.9)
M. catarrhalis	23				23 (4.9)
H. influenzae	45	2			47 (10.0)
H. parainfluenzae	37				37 (7.9)
S. aureus	19				19 (4.1)
P. aeruginosa	8				8 (1.7)
K. pneumoniae	9				9 (1.9)
K. oxytoca	3				3 (0.6)
E. coli	16	1			16 (3.4)
S. marcescens	3				3 (0.6)
Other Enterobacteriaciae	8				8 (1.7)
Other gram negatives	16				16 (3.4)
Contaminants		18			18 (3.8)
M. pneumoniae				23	23 (4.9)
C. pneumoniae				1	1 (0.2)
B. pertussis				2	2 (0.4)
Adenovirus				1	1 (0.2)
Rhinovirus				55	55 (11.7)
Influenzavirus				38	38 (8.1)
Parainfluenzavirus				11	11 (2.3)
hMPV				15	15 (3.2)
RSV				12	12 (2.6)
Coronavirus				10	10 (2.1)
Total	235	57	72	168	496

a UAT = Urinary Antigen Test, hMPV = Human Metapneumovirus, RSV = Respiratory Syncytial Virus

eTable 5.2 Overview of infections and co-infections

	N (% of total patients)
1 bacterial pathogen	117(25)
2 bacterial pathogens	31 (6.6)
3 bacterial pathogens	8 (1.7)
4 bacterial pathogens	0 (0)
5 bacterial pathogens	1 (0.2)
1 viral pathogen	55 (11.7)
2 viral pathogens	1 (0.2)
1 bacterial and 1 viral pathogen	49 (10.5)
2 bacterial and 1 viral pathogen	27 (5.8)
3 bacterial and 1 viral pathogen	4 (0.8)
4 bacterial and 1 viral pathogen	3 (0.6)
1 bacterial and 2 viral pathogen	1 (0.2)
Total	297 (63.5)

eTable 5.3 New antibiotic prescriptions from day 1 till 30±2

	Control group, n=156	CRP group, n=156	PCT group, n=156
New antibiotic prescriptions			
Number of cases (%)	34 (21.8)	35 (22.4)	52 (33.3)
HR (95% CI)	Reference	1.05 (0.66 - 1.68) p = 0.84	1.65 (1.07 - 2.55) p = 0.023

eTable 5.4 Reasons for new antibiotic prescriptions in the intervention period

	Control, n (% of group)	PCT, n (% of group)	CRP, n (% of group)	Total, n (% of all patients)
Recurring fever/new infection	10 (6.4)	21 (13.5)	12 (7.7)	43 (9.2)
Persisting fever	0 (0)	4 (2.6)	3 (1.9)	7 (1.5)
Empyema	8 (5.1)	0 (0)	1 (0.6)	9 (1.9)
Fear of under treatment despite good clinical recovery	3 (1.9)	3 (1.9)	1 (0.6)	7 (1.5)
Insufficient clinical recovery	1 (0.6)	2 (1.3)	2 (1.3)	5 (1.1)
Other infection	1 (0.6)	3 (1.9)	1 (0.6)	5 (1.1)
Pathogen resistant to empiric therapy	1 (0.6)	2 (1.3)	2 (1.3)	5 (1.1)
Cryptogenic Organizing Pneumonia	0 (0)	0 (0)	1 (0.6)	1 (0.2)
Total n (%)	24 (15.4)	35 (22.4)	23 (14.7)	82 (17.5)

eTable 5.5 Excluded patients not eligible for the study intervention

Reason	Total (n)
Normal chest X-ray	188
Known malignancy	72
Immunocompromised	51
Aspiration pneumonia	42
Hospital acquired pneumonia	37
Concomitant infection	25
Empyema on admission	12
Total	427

eTable 5.6 Sensitivity analysis for the primary outcome excluding patients that developed empyema during the intervention period

	Control group, n=148	CRP group, n=155	PCT group, n=156
Sensitivity analysis RR (95% CI)	Reference	0.74 (0.64 - 0.85) p < 0.001	0.83 (0.73 - 0.95) p = 0.005

CASE SUMMARIES

all patients with a new antibiotic prescription after a shorter initial course of antibiotics based on the biomarker algorithms

n = 30 PCT group
n = 11 CRP group

5 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment with amoxicillin (=admission day 4) based on PCT levels. Microbiology results yielded B. pertussis. Developed fever the next day (=admission day 5) and was treated for a hospital acquired pneumonia. CRP admission day one 201, PCT 0.08. CRP on admission day four 54, PCT 0.06 CRP on admission day five 83. Was treated with antibiotics for an additional 12 days.

19 – CRP group
Antibiotics stopped on admission day 5, after 4 days of treatment based on CRP levels. CRP on admission day one 75, on admission day five 26. Antibiotics were restarted on admission day 7 due to insufficient recovery with a sputum culture yielding a H. influenza. Patient received antibiotics for an additional 38 days.

26 – PCT group
Antibiotics stopped on admission day 5, after 4 days of treatment based on PCT levels. PCT on admission day one 1.59, on admission day five 0.15. On day 24 patient presented with an acute exacerbation of COPD with signs of a lower respiratory tract infection and received antibiotics for an additional 7 days.

32 – PCT group
Antibiotics stopped on admission day 5, after 4 days of treatment based on PCT levels. Patient had a persisting fever at that point, but his clinical condition and clinical parameters all improved. Antibiotics were restarted the next day due to fear of under treatment. PCT on admission day one was 0.22, on admission day five 0.05. Microbial tests only yielded a parainfluenzavirus. Patient received antibiotics for an additional 8 days.

34 – PCT group
Antibiotics stopped on admission day 5, after 4 days of treatment based on PCT levels. Antibiotics were restarted the next day due to purulent sputum, sub-febrile temperature and a CRP of 360. Sputum culture yielded S. pneumonia, M. Catarrhalis, H. parainfluenzae. CRP on admission day 1 was 336, PCT 10.30 On admission day five PCT was 0.11. Patient received antibiotics for an additional 7 days.

36 – PCT group
Antibiotics stopped on admission day 4, after 3 days of treatment based on PCT levels. Antibiotics were restarted on admission day 8 due to fever that was attributed to the CAP. Microbial tests yielded no pathogens. PCT on admission day one 0.05, CRP 1. On admission day 4 PCT 0.06, On admission day 5 CRP was 82. Patient received antibiotics for an additional 11 days.

53 – CRP group
Antibiotics stopped on day 4, after 3 days of treatment based on CRP levels. Microbial tests only yielded an influenzavirus. CRP on admission day one 50 on admission day four 14. Patient was discharged on admission day 7 and took additional antibiotics she had at home for 5 days due to complaints of increased cough and sputum volume. She was eventually readmitted 5 days later with sputum culture yielding a P. aeruginosa which the previous antibiotics did not cover, so she received additional antibiotic treatment for 20 days.

56 – CRP group
Antibiotics stopped on admission day 5, after 4 days of antibiotic treatment based on CRP levels. Antibiotics restarted 3 days later due to an increase in dyspnea, sputum volume with sputum culture yielding a P. Aeruginosa. CRP on admission day one 32, on admission day five 6. Patient received additional antibiotic treatment for 7 days.

58 – PCT group
Antibiotics stopped on admission day 6, after 5 days of treatment based on PCT levels. Microbial tests yielded S. pneumoniae(urine antigen test), enterobacter spp. (sputum culture) and rhinovirus (oralpharyngeal swab). PCT on admission day one 4.9, on admission day six 0.40. Patient had fully recovered clinically by that point and was discharged. He presented himself to our ER department 5 days later with chest pains. A pulmonary embolism was ruled out, CRP was 12 and he was discharged without additional therapy. He was then re-admitted 30 days after the initial admission with meningitis/encephalitis and received additional antibiotics for 4 days until he died.

65 – PCT group
Antibiotics stopped on admission day 4, after 3 days of treatment based on PCT levels. On the outpatient visit on day 32 patient presented with signs of a lower respiratory tract infection and received additional antibiotics for 7 days.

85 – CRP group
Antibiotics stopped on admission day 4, after 3 days of treatment based on CRP levels. Antibiotics were restarted on admission day 5 due to recurring fever. Microbial tests yielded no pathogens. CRP on admission day one 164, on admission day four 82, on admission day five 118. Antibiotics were restarted for 7 days, patient recovered and was discharged on admission day 14. He was then re-admitted a week later with an exacerbation of COPD due to a recurring pneumonia and antibiotics were restarted for another 7 days. In total he received additional antibiotics for 14 days.

95 – PCT group
Antibiotics stopped on admission day 4, after 3 days of treatment based on PCT levels. Microbial tests yielded no pathogens. Patient recovered and was discharged on admission day 4. Nine days later patient was readmitted with an aspiration pneumonia and treated for an additional 7 days.

132 – PCT group
Antibiotics stopped on admission day 6, after 5 days of treatment based on PCT levels. Patient recovered quickly and was discharged on admission day 5. A pathogen was never identified. PCT on admission day one 3.89 on admission day six 0.39. 17 days later patient was prescribed an additional 7 days of antibiotics due to an otitis media.

138 – PCT group
Antibiotics stopped on admission day 4, after 3 days of treatment based on PCT levels. Oropharyngeal PCR yielded M. pneumoniae. Patient was discharged on admission day 10. PCT on admission day one 0.10 on admission day four 0.05. He was readmitted 19 days after discharge due to a urinary tract infection associated with an indwelling catheter and received antibiotics for an additional 28 days.

141 – CRP group
Antibiotics stopped on admission day 6, after 5 days of treatment based on CRP levels. Microbial tests yielded a H. parainfluenzae. Patient was discharged on admission day 5. He was readmitted 13 days later with heart failure with possible signs of a lower respiratory tract infection and received additional antibiotics for 7 days.

169 – PCT group
Antibiotics stopped on admission day 4, after 3 days of treatment based on PCT levels. Microbial tests yielded a H. parainfluenzae, K. oxytoca and human metapneumovirus. PCT on admission day one 0.07 on admission day four 0.05. Patient was recovering slowly even after stopping antibiotics, but due to a resistant pathogen for the empiric antibiotic treatment and prolonged clinical recovery (dyspnea, need for supplemental oxygen and sputum retention) extra antibiotics were started on admission day 11 for an additional 7 days. Patient was eventually discharged on admission day 17.

179 – PCT group
Antibiotics stopped on admission day 4, after 3 days of treatment based on PCT levels. Microbial tests yielded a M. pneumoniae. PCT on admission day one 0.19 on admission day four 0.05. CRP on admission day one 130 on admission day five 79. Empiric treatment happened to cover mycoplasma spp in this patient. However, the day the antibiotics were discontinued, the PCR came back positive and antibiotics were restarted by the treating physician due to fear of possible undertreatment, despite a good clinical recovery at that point. He was discharged on admission day 8. Patient received additional antibiotics for 21 days.

192 – CRP group
Antibiotics stopped on admission day 4, after 3 days of treatment based on CRP levels. Microbial tests yielded H. Influenzae and rhinovirus. Patient was discharged on admission day 9. CRP on admission day one 97 on admission day four 13. Antibiotics were restarted on admission day 6 due to recurring signs of a lower respiratory tract infection. Patient received additional antibiotic therapy for 7 days.

212 – CRP group.
Antibiotics stopped on admission day 7, after 6 days of treatment based on CRP levels. Microbial tests yielded a S. Aureus (sputum culture) and a human metapneumovirus. CRP on admission day one 22 on admission day seven 9. Patient recovered after initial treatment and was discharged on admission day 7. She was then readmitted 15 days later with recurring symptoms indicative of a lower respiratory tract infection/pneumonia and received antibiotics for an additional 7 days.

213 – CRP group
Antibiotics stopped on admission day 4, after 3 days of treatment based on CRP levels. Microbial tests yielded no pathogens. CRP on admission day one was 54 CRP on admission day four 19. Patient recovered quickly and was discharged on admission day 5. Patient was readmitted 8 days later due to an exacerbation of COPD with signs of a lower respiratory tract infection and received antibiotics for an additional 5 days.

220 – PCT group
Antibiotics stopped on admission day 4, after 3 days of treatment based on PCT levels. Microbial tests yielded no pathogens. PCT on admission day one 0.21 on admission day four 0.23. Patient was discharged on admission day 11. Patient was recovering slowly, but due to a persisting fever antibiotics were restarted on admission day 8 also covering atypical pathogens.

221 – PCT group
Antibiotics stopped on admission day 6, after 5 days of treatment based on PCT levels. Microbial tests yielded respiratory syncytial virus. PCT on admission day one 4.01 on admission day six 0.20. Patient was discharged on admission day seven. 18 days later he was readmitted due to recurring pneumonia and received an additional 7 days of antibiotic treatment.

232 – PCT group
Antibiotics stopped on admission day 6, after 5 days of treatment based on PCT levels. Microbial tests yielded S. pneumoniae, E. Coli and parainfluenzavirus. PCT on admission day one 0.64 on admission day six 0.11. Patient was discharged on day 6. On the outpatient visit on day 28 his chest x-ray barely improved and he had signs of a lower respiratory tract infection/pneumonia. He received an additional 7 days of antibiotics.

242 – PCT group
Antibiotics stopped on admission day 6 after 5 days of treatment based on PCT levels. Microbial tests yielded K. pneumoniae. PCT on admission day one 4.92 on admission day six 0.25. Patient was discharged on admission day 7. Patient was readmitted 14 days later with a septic shock, was admitted to the ICU and received additional antibiotics for 15 days.

250 – PCT group
Antibiotics stopped on admission day 7 after 6 days of treatment based on PCT levels. Microbial tests yielded S. pneumoniae. PCT on admission day one 3.62 on admission day seven 0.22. Patient was discharged on admission day 6. Patient was readmitted with a non-infectious exacerbation of COPD 10 days later and received no antibiotics, recovered and was discharged 5 days later. 4 days later (25 days after the initial admission) she presented to the ER department with clinical signs of recurring pneumonia, was admitted and treated for a hospital acquired pneumonia. She received additional antibiotic therapy for 7 days.

260 – PCT group
Antibiotics stopped on admission day 5 after 4 days of treatment based on PCT levels. Microbial tests yielded S. pneumoniae, S. Aureus (sputum culture) and rhinovirus. The same day antibiotics were restarted in the evening shift due to concerns of under treatment. The next morning antibiotics were again discontinued by the treating physician. Patient received 1 extra day of antibiotics.

279 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded no pathogens. PCT on admission day one 0.13 on admission day four 0.08.
Antibiotics were restarted 2 days later due to recurring fever. Patient received an additional 6 days of antibiotics.

320 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded no pathogens. PCT on admission day one 0.10 on admission day four 0.10.
On admission day 9 antibiotics were restarted due to recurring signs of a lower respiratory tract infection. Patient received an additional 14 days of antibiotic treatment.

331 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded H. Influenzae. PCT on admission day one 0.13 on admission day four 0.13. CRP on admission day one 197, on admission day five 134. Antibiotics were restarted the same day due to persistent fever and fear of an atypical pathogen. Patient received an additional 9 days of antibiotics.

348 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded rhinovirus. PCT on admission day one 0.19 on admission day four 0.12. CRP on admission day one 194 and on admission day five 174. Antibiotics were restarted on admission day 5 due to persistent fever. Despite the fever and slowly declining biomarker levels all other symptoms improved and patient was set to be discharged on admission day 6. On the day of her discharge she was found dead in her room due to aspiration of food leading to asphyxia. Patient received an additional day of antibiotics.

378 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded M. Catarrhalis and rhinovirus. PCT on admission day one 0.73 on admission day four 0.20. Patient visited his general practitioner 17 days after antibiotics were discontinued with complaints of fever and increased sputum volume. Patient received an additional 7 days of antibiotics.

395 – CRP group
Antibiotics stopped on admission day 5, after 4 days of treatment based on CRP levels. Microbial tests yielded S. pneumoniae and influenzavirus. CRP on admission day one was 130 CRP on admission day five 43. After stopping of antibiotic treatment patient had a recurring fever on the same day and received an additional 6 days of antibiotic treatment.

396 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded H. Influenzae, S. Aureus (sputum culture) and M. pneumoniae. PCT on admission day one 0.12 on admission day four 0.05. Patient was discharged on admission day 5 and presented himself to the ER department 6 days later complaining of dyspnea but no other symptoms and received an additional 7 days of antibiotic treatment targeting the M. pneumoniae.

399 – CRP group
Antibiotics stopped on admission day 4, after 3 days of treatment based on CRP levels. Microbial tests yielded S. pneumoniae and human metapneumovirus. CRP on admission day one was 236 CRP on admission day four 85. During the outpatient visit on day 32 patient presented with signs of a lower respiratory tract infection and received an additional 7 days of antibiotic treatment.

403 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded H. Influenzae. PCT on admission day one 0.19 on admission day four 0.11. Patient was recovering and clinical signs of pneumonia were improving, but remained oxygen dependent for some time. On admission day 11 she had a worsening hypoxia and a recurring fever and was treated for a Hospital-acquired pneumonia. She received 10 days of additional antibiotic treatment.

405 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded S. Aureus (sputum culture) and influenzavirus. PCT on admission day one 0.87 on admission day four 0.15. Patient recovered and was discharged on admission day 6. Ten days after discharge patient phoned the outpatient clinic complaining of dyspnea and fatigue. The S. Aureus in the sputum culture was resistant for the empiric antibiotic coverage she received earlier. Patient received an additional 7 days of antibiotic treatment targeting the S. Aureus.

409 – PCT group
Antibiotics stopped on admission day 5 after 4 days of treatment based on PCT levels. Microbial tests yielded S. pneumoniae and influenzavirus. PCT on admission day one 0.97 on admission day five 0.15. Patient was discharged on admission day 5. Two days after discharge she presented to another hospital with persisting dyspnea and was readmitted. She received an additional 7 days of antibiotic treatment.

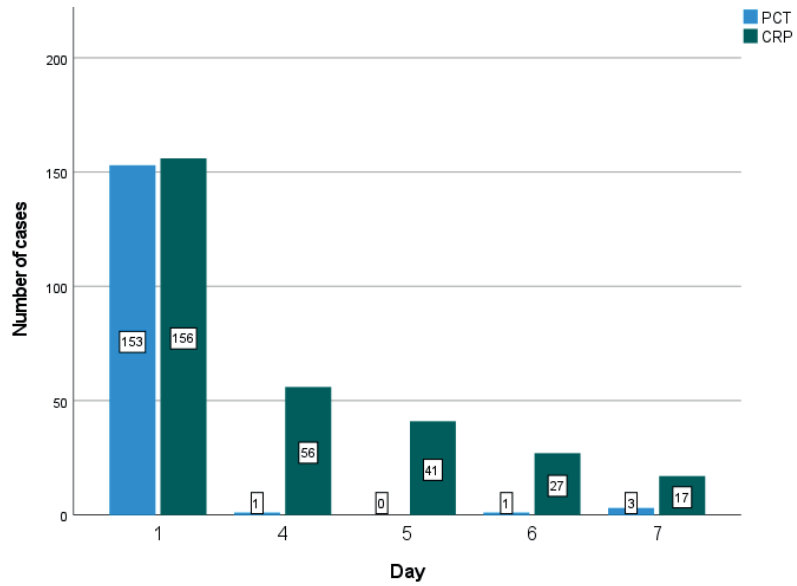
415 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded S. pneumoniae, H. parainfluenzae and influenzavirus. PCT on admission day one 0.24 on admission day four 0.05. Patient was recovering and was discharged on admission day 4, but had a recurring fever prior to discharge. The treating physician chose to continue antibiotic treatment for another 6 days.

416 – CRP group
Antibiotics stopped on admission day 4 after 3 days of treatment based on CRP levels. Microbial tests yielded no pathogens. CRP on admission day one 77 PCT 0.08 on admission day four CRP was 29. Patient was discharged on admission day 4. Sixteen days after discharge patient had recurring signs of a lower respiratory tract infection and received an additional 7 days of antibiotic treatment.

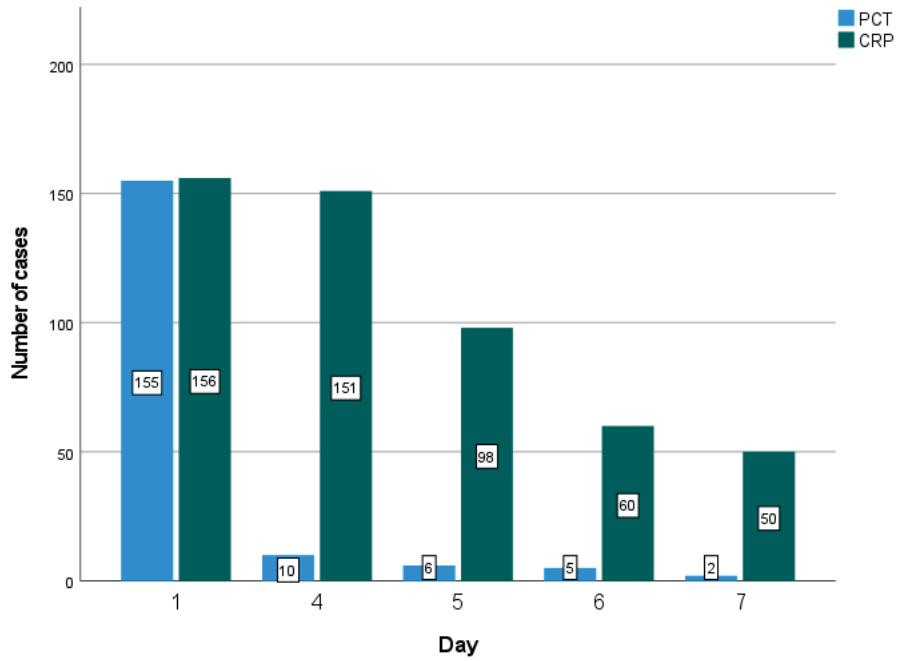
432 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded M. pneumoniae. PCT on admission day one 0.09 on admission day four 0.06. Patient was recovering well with empiric antibiotic treatment that did not cover the M. pneumoniae. When the M. pneumoniae was found the treating physician decided to treat the patient for another 15 days specifically targeting the M. pneumoniae due to fear of under treatment. Patient was discharged on admission day 4.

444 – PCT group
Antibiotics stopped on admission day 4 after 3 days of treatment based on PCT levels. Microbial tests yielded H. Influenzae and E. Coli. PCT on admission day one 0.21 on admission day four 0.19. Patient recovered and was discharged on admission day 4. Three days later patient had recurring signs of a lower respiratory tract infection, was re-admitted and treated for another 6 days for a hospital acquired pneumonia. He died due to unknown causes on day 23, eleven days after completion of his treatment for the hospital acquired pneumonia.

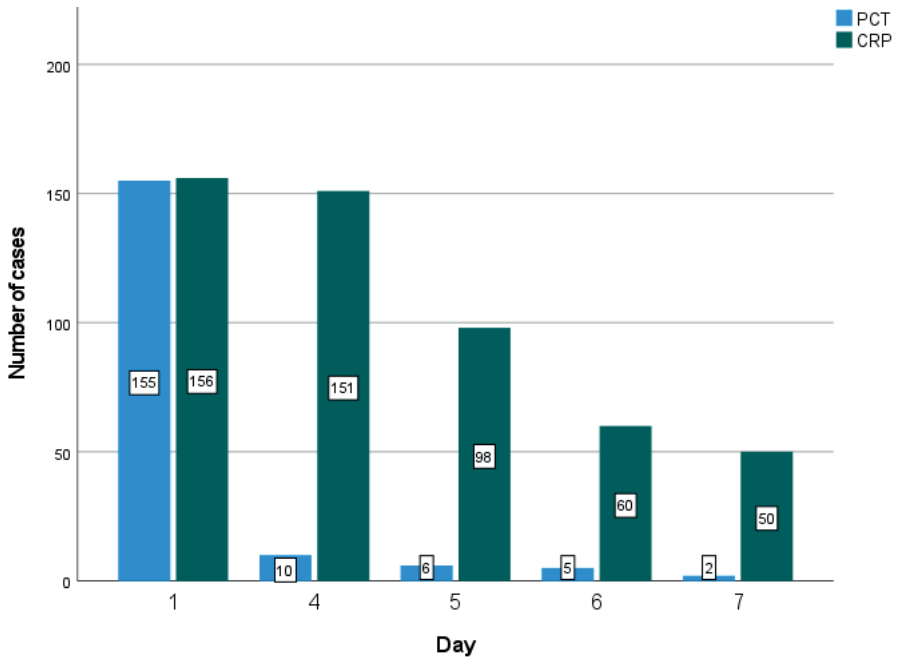
eFigure 5.1 Overview of biomarker assessment per day in the control group



eFigure 5.2 Overview of biomarker assessment per day in the CRP group

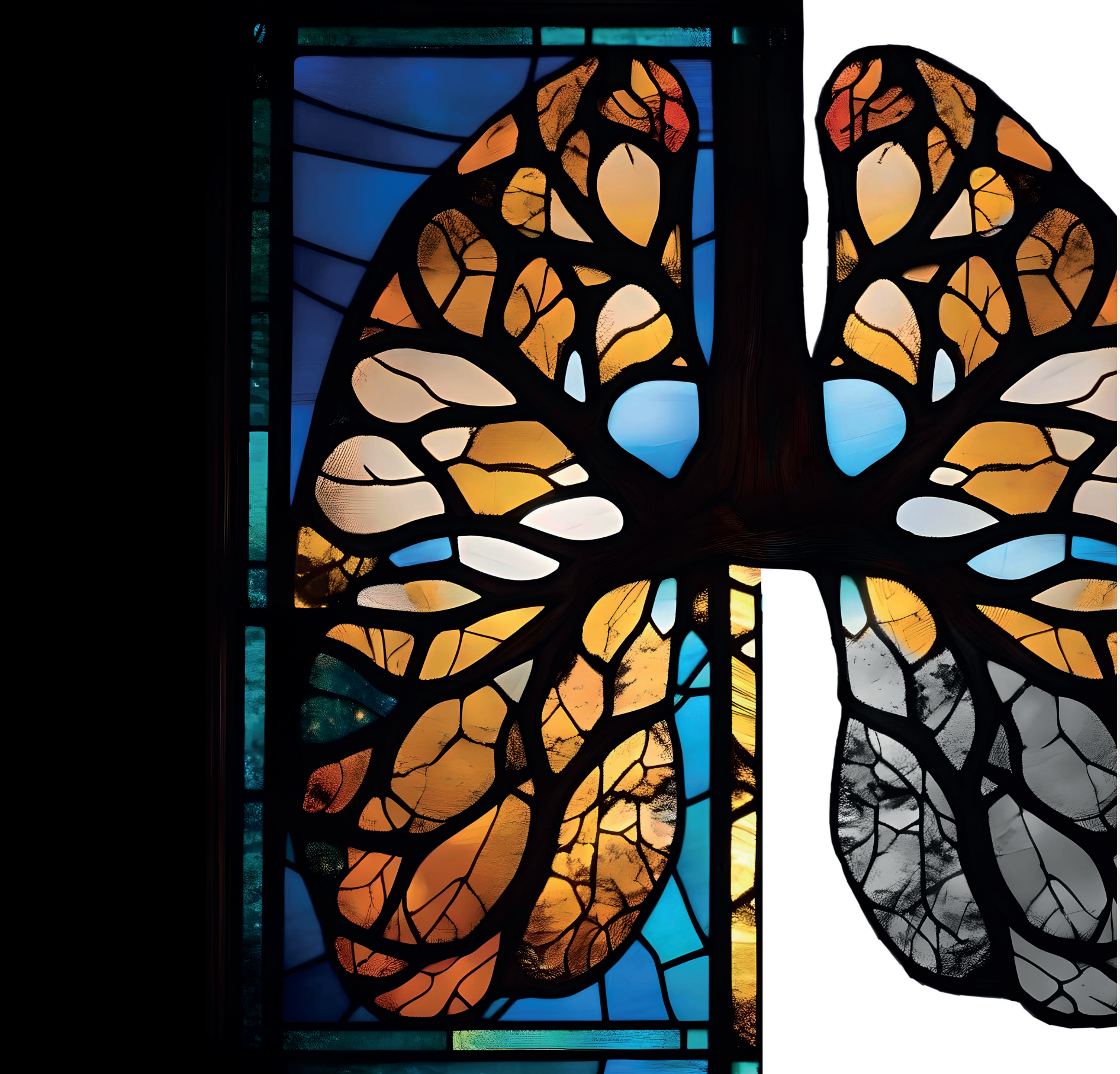


eFigure 5.3 Overview of biomarker assessment per day in the PCT group



eResults 5.1 Overview of concordance between CRP and PCT assessments on day 4 in the PCT group

	Day 4	Algorithm overruled/influenced by CRP
Both stop criteria reached, values concordant	n=24	No
CRP low enough, but PCT criteria not reached	n=6	Antibiotics continued in all 6 patients
PCT low enough but CRP criteria not reached	n=10	Antibiotics stopped in all 10 patients



Chapter 6

MR-proADM and adverse outcomes in patients with Community Acquired Pneumonia: a matched case-control study

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ABSTRACT

Objective

In patients with Community-Acquired Pneumonia (CAP) midregional proadrenomedullin (MR-proADM) has been proposed as a biomarker to enhance risk stratification and predict negative outcomes. We aimed to determine differences in MR-proADM levels on admission between matched cases and controls, focusing on short-term adverse outcomes, including treatment failure, short-term mortality, and re-admission after discharge in patients admitted with CAP to a non-ICU hospital ward. We used material from the REDUCE study (NCT01964495), first registered 2013-okt-14.

Results

In this matched case-control study of patients hospitalized with CAP on non-ICU wards MR-proADM levels were comparable between developing and not developing negative outcomes. MR-proADM values increased with increasing CURB-65 score, with $p < 0.001$. These findings do not support the usefulness of MR-proADM as a prognostic variable in patients hospitalized with CAP.

INTRODUCTION

Community-acquired pneumonia (CAP) is an important cause of death worldwide.
In Europe ~3.3 million people develop CAP per year, of whom approximately 10% need hospital admission[1].
Risk stratification for patients presenting to emergency departments with CAP is important to determine the optimal care strategy, including decisions for admission to hospital wards, ICU or ambulatory care. Clinicians frequently use risk scores such as the CURB-65 and PSI score, which have been developed for predicting CAP-associated short-term mortality[2,3]. Yet, the PSI-score is largely age-driven and tends to overestimate severity in elderly patients[4], and the CURB-65 score has a relatively low sensitivity for predicting admission to the ICU or critical care interventions[5].

Multiple biomarkers have been tested for their capacity to improve prognosis prediction in patients with CAP. Both CRP and PCT have proven useful to predict typical bacterial pathogens, assessing severity, predicting mortality risk and potential complications of CAP[6-8].

One of the less frequently tested biomarkers is midregional proadrenomedullin (MR-proADM), which is a fragment of the precursor of adrenomedullin. Adrenomedullin is synthesized during severe infections and has vasodilatory, immune modulatory and antimicrobial activity[9].

In several studies MR-proADM appeared as a useful tool for risk stratification in CAP patients, enhancing accuracy of existing risk scores, independent of etiology and able to predict complications and possibly long term outcomes[10-12].

Yet in a randomized trial among patients presenting to the emergency department with lower respiratory tract infections a strategy to triage and discharge patients based on medical and biopsychosocial risk assessment in conjuction with MR-proADM levels failed to reduce length of stay and adverse outcomes, compared to a strategy not using MR-proADM. However, the study algorithm was overruled in 39.3% of patients at presentation and in 34.5% during hospitalization[13].

Whether use of MR-proADM improves patient outcomes is yet to be determined and an optimal cut-off value for MR-proADM is not known. Most studies using MR-proADM have focused on predicting mortality or complications from CAP requiring ICU-admission. This might diminish the prognostic usefulness of MR-proADM for patients admitted to regular hospital wards with regards to other relevant outcomes.
We, therefore, aimed to determine differences in MR-proADM levels on admission between matched cases and controls, focusing on short-term adverse outcomes, including treatment failure, short-term mortality, and re-admission after discharge in patients admitted with CAP to a non-ICU hospital ward.

METHODS

Study design and population

We conducted a matched case-control study using data from a prospective randomized controlled trial focusing on biomarker guided antibiotic stewardship in patients with radiologically proven CAP hospitalized to a regular ward (REDUCE study). The study was performed in three large teaching hospitals in the Netherlands: the North West Hospital Group in Alkmaar, the Slotervaart hospital in Amsterdam and the ISALA clinics in Zwolle. The study protocol was approved by the Medical Ethics Committee associated with the Northwest hospital and is in full compliance with the Helsinki declaration. The study protocol was registered in the clinicaltrials.gov database (NCT01964495).
Blood samples obtained from patients with CAP admitted to the North West hospital in Alkmaar between December 2013 and March 2015 were used for the current analysis.

Cases were defined as patients with at least 1 negative outcome, which included need for extra antibiotics, development of empyema, need of ICU admission, mortality within 30 days or re-hospitalization. Cases were matched on gender, age and Charlson comorbidity index to 1 control patient from the same cohort that did not experience a negative outcome.

Outcomes

We determined differences between MR-proADM levels on admission between cases and controls, as well as associations between MR-proADM levels on admission and severity of pneumonia based on CURB-65 score. All data was prospectively collected during the REDUCE study.

Laboratory assessments

MR-proADM levels were measured in EDTA plasma samples by an immunoluminometric sandwich assay (BRAHMS MR-proANP LIA,BRAHMS AG). Thirty samples were measured in one run and all samples consisted of at least 26 µl, which was required to measure the concentration of MR-proADM. The limit of quantitation (LoQ) was 0.23 nmol/L and the functional assay sensitivity (FAS) 0.25 nmol/L. Furthermore, the intra-assay Coefficients of Variability was found to be 3.43% and the inter-assay Coefficient of Variability was found to be 8.24%.

Statistics

All cases were matched using R with regards to gender, age with a tolerance of 5 years and Charlson weighted index of comorbidity with a tolerance of 2 points. Each case was then matched with 1 control.
Differences at baseline between cases and controls were analyzed using t-tests, chi-square tests and Mann-Whitney U tests where appropriate.
MR-proADM levels were assessed through conditional logistic regression, incorporating stratified bootstrapping to accommodate the matched pairs. IBM SPSS Statistics for Windows, version 28, was utilized for analysis.

Results

In a cohort of 156 patients, 47 met the criteria for an adverse outcome (cases), of which two could not be matched to suitable controls and in 2 blood samples were lost. Consequently, 45 cases were matched to 45 controls (Supplementary figure S6.1). Cases and controls were comparable for smoking status, severity of pneumonia according to CURB65 score and etiology of pneumonia (Table 6.1). Mean MR-proADM concentrations on admission were 1.35 nmol/L (SD ±0.60) and 1.33 nmol/L (SD ±0.11) for cases and controls, respectively, yielding an odds ratio (OR) of 1.044 (95% CI 0.57 - 1.92), p = 0.89. Furthermore, MR-proADM levels were comparable for cases and controls in the different subcategories of adverse outcomes (table 6.2). MR-proADM values increased with increasing CURB-65 score, with p < 0.001 (Figure 6.1).

Figure 6.1

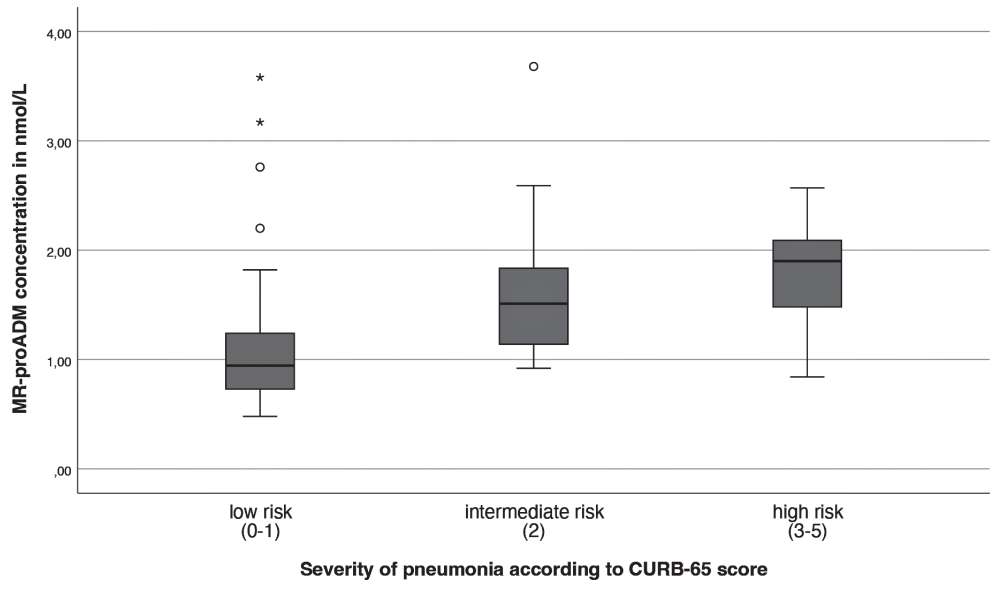

Table 6.1 Baseline characteristics

	Cases, n = 45	Controls, n = 45	p-value
Age, mean (±SD)	70.27 (±14.31)	70.13 (±14.61)	0.97
Gender: males, n (%)	25 (55.6)	25 (55.6)	1.00
Charlson weighted index: co-morbidity, median (IQR)	1 .00 (0.50 – 2.00)	1.00 (0.00 – 2.00)	0.80
Smoking, n (%)			0.40
Current smoker	8 (17.8)	14 (31.1)	
Previous smoker	25 (55.6)	22 (48.9)	
Never smoked	10 (22.2)	9 (20.0)	
Unknown	2 (4.4)		
CURB-65, median (IQR)	1.00 (1.00 – 2.00)	1.00 (1.00 – 2.00)	0.72
Etiology of pneumonia, n (%)			0.63
Bacterial	22 (51.1)	18 (40)	
Viral	3 (6.7)	5 (11.1)	
Bacterial and viral	14 (31.1)	18 (40.0)	
Unknown	5 (11.1)	4 (8.9)	

Table 6.2 MR-proADM levels in different subsets of cases compared to their corresponding controls

	Cases	Controls	p-value
MR-proADM on admission	1.35 ±0.60	1.33±0.11	0.89
Events			
Treatment failure, n=29	1.31 ±0.55	1.35 ±0.70	0.82
Rehospitalization, n=13	1.36 ±0.76	1.25 ±0.90	0.71
Mortality, n=3	1.64 ±0.69	1.44 ±1.00	0.74

DISCUSSION

In this matched case-control study of patients hospitalized with CAP on non-ICU wards MR-proADM levels were comparable between developing and not developing negative outcomes. These findings do not support the usefulness of MR-proADM as a prognostic variable in patients hospitalized with CAP.

Most prior studies have focused on the value of MR-proADM as a substitute for or an enhancement to existing risk scores. Our findings did confirm results of several other studies that MR-proADM levels were associated with CURB-65 scores[14-17]. Moreover, several studies and a meta-analysis demonstrated that elevated MR-proADM levels were associated with a higher risk of mortality and cardiovascular events[11,15,17.] Since we studied patients admitted to non-ICU wards, mortality in our population was low precluding meaningful comparisons with MR-proADM. Moreover, among patients presenting to the emergency department with lower respiratory tract infections Albrich et al. found a significant association between biomarkers and ICU-admission and empyema[14.] Similarly, Bello et al. found an association between a wide variety of possible complications that included cardiac failure, renal failure, septic shock and new hyperglycaemia as well as pulmonary complications such as empyema, pleural effusion or respiratory failure with or without acidosis[15.]
A third study reported a statistically significant correlation between MR-proADM and respiratory failure/shock and need of ICU admission in patients with CAP[18].
However, less is known about associations between MR-proADM levels and treatment outcomes in patients hospitalized in non-ICU wards. Multiple studies have been performed with multiple endpoints, yielding different results[13,19,20].

Our study's matched case-cohort design does come with some inherent limitations regarding variability and generalizability to the broader CAP population, especially since our sample size is relatively small and 30-day mortality is relatively low. Furthermore, the design inherently precludes definitive statements regarding the predictive value of MR-proADM in this context.
However, it possesses a notable strength: we conducted matching within the same CAP population, distinguishing our approach from other studies that paired pneumonia patients with individuals admitted to different hospital wards. As a result, it enhances the comparability of our cases and controls, providing a clearer framework for evaluating differences in MR-proADM and negative outcomes. Since all outcomes were prospectively recorded the risk of attention or recall bias is lower than if outcomes were retrospectively assessed.

Overall our results do not support MR-proADM as a prognostic variable once the patient has been hospitalized. Future studies should primarily focus their efforts on developing prospective clinical algorithms to determine if MR-proADM could or should impact the treatment of admitted patients.

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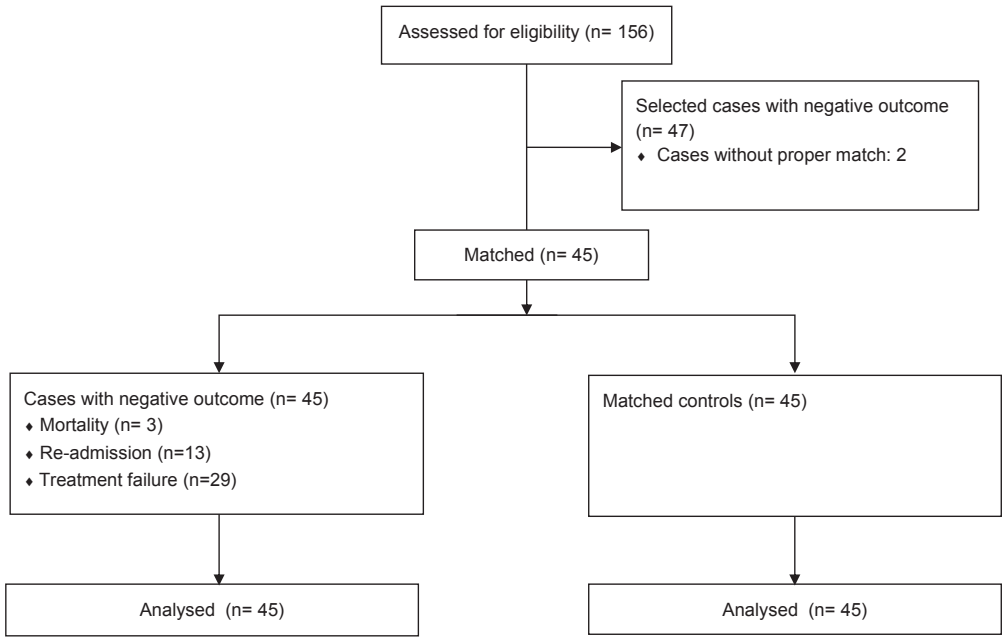
Figure S6.1


Assessed for eligibility (n= 156)
Selected cases with negative outcome (n= 47)
♦ Cases without proper match: 2
Matched (n= 45)
Cases with negative outcome (n= 45)
♦ Mortality (n= 3)
♦ Re-admission (n=13)
♦ Treatment failure (n=29)
Matched controls (n= 45)
Analysed (n= 45)
Analysed (n= 45)

Chapter 7

General discussion

GENERAL DISCUSSION

In this thesis different aspects of diagnosis and management of CAP in adults are discussed. The following sections summarize and discuss the development, validation and potential role of a real-time quantitative PCR in diagnosing pneumococcal pneumonia, the validation and performance of a *Legionella* prediction score, potential of a cytokine panel to distinguish different aetiologies of pneumonia, the performance of a CRP-guided and PCT-guided treatment algorithm in reducing antibiotic treatment duration and MR-proADM concentrations in patients with and without negative outcomes. In each section recommendations for clinical practice/future research are given.

Diagnosing pneumococcal infection

Diagnosing pneumococcal pneumonia can be challenging. The combination of urinary antigen testing, blood cultures and sputum cultures is considered as the gold standard for microbiological work-up. However, this poses a significant diagnostic challenge since approximately 30-35% of patients in the Netherlands are pre-treated by GP's with antibiotics and this significantly reduces the yield of conventional culture methods[1,2]. PCR techniques targeting pneumococcal genes have the potential to increase diagnostic yield and potentially differentiate colonization from infection.

In **chapter 2** we describe the development, validation and performance in a select subset of patients of a real-time quantitative PCR (rt-qPCR) targeting the *lytA* gene present in pneumococci. In vitro the *lytA* quantitative PCR appeared a reliable test to detect *S. pneumoniae* with sensitivity and specificity of 100%. Based on this, the test hold promise for being used in vivo, where it could distinguish between infection and colonization. In a small sample of patients with complete diagnostic work-up sensitivity and specificity for *S. pneumoniae* were 72.7 and 84.6%, respectively, when using a cutoff value of 6.000 DNA copies/mL. The in-vitro lower limit of detection (LLOD) turned out to be between approximately 1 and 10 copies/µL, which is similar to the LLOD's found in other studies varying from <10 copies per reaction to 4.3 copies per reaction[3-5]. This LLOD makes the differentiation between colonization and infection possible. Other studies also reported excellent in-vitro sensitivity and specificity using a rt-qPCR targeting the *lytA* gene[4,6,7]. In one study sensitivity and specificity were 100% using 70 positive controls and 9 non-pneumococcal streptococci (including 2 *Streptococcus mitis* strains)[7]. This 100% specificity was confirmed by another study using 23 non-pneumococcal streptococci (including three that closely resemble *S. pneumoniae*; 2 *S.oralis* strains and 1 *S. mitis* strain)[6]. In the largest study a total of 257 strains were tested, belonging to 37 different species including 30 *S. mitis* strains, with no false negative results and only one false positive result out of 30 *S. mitis* strains. This sample was also tested positive by two rapid pneumococcal antigen tests (Wellcogen and Phadebact)[4.]

A study using the same positive control (ATCC 49619), primers and probe, tested 23 *S. pneumoniae* strains and 29 negative controls (including six non-pneumococcal species, one being *S. mitis*). Testing these non-pneumococcal strains makes for a valuable contribution to previous trials because they generate signals reported specific to *S. pneumoniae*, in terms of optochin susceptibility, bile solubility, and Quellung reaction, which are the classic methods used to identify pneumococci[8]. Our PCR was able to discriminate between these strains and *S. pneumoniae*. However, the small number of strains tested might overestimate specificity.

Our pilot-study contained only a small number of patients admitted with either pneumococcal pneumonia or CAP caused by other pathogens. This pilot-study was conducted to perform a preliminary in vivo validation of the qPCR and was not designed as a full clinical trial. Preliminary results are promising: best AUC of 0.714 with a sensitivity of 57.1% and specificity of 85.7% with a cut-off value of 6.000 copies/mL. The AUC was even higher when only using the samples of patients with a complete diagnostic workup; a sensitivity of 72.7% and a specificity of 84.6% using a cut-off value of 6.000 copies/mL (AUC 0.787). In three patients without detection of *S. pneumoniae* using the current diagnostic standard where a virus was detected (two coronaviruses and one influenza A virus) concentrations of *S. pneumoniae* were above 40.000 copies/mL. The most likely explanation for these high concentrations of DNA copies/mL is false-negative results of the current pneumococcal tests, but it limited the specificity in the pilot study. Only one of these patients was pretreated with antibiotics and all had a favourable outcome with amoxicillin. Given the high DNA concentrations above the cut-off values for colonization and the favourable response to therapy an underlying pneumococcal infection seems very likely.

In a clinical study of HIV-infected patients the use of the *lytA* rt-qPCR with a cut-off value of 8.000 copies/mL to diagnose pneumococcal pneumonia on nasopharyngeal (NP) swabs had sensitivity and specificity of 82.2% and 92%, respectively. The proportion of CAP cases attributable to pneumococci increased from 27.1% to 52.5%[9]. In another study using NP samples, the optimal cut-off was 2351 copies/mL, which yielded a sensitivity of 83.3% and specificity of 80.9% for pneumococcal CAP[10]. However studies done to date are highly heterogeneous, including children and adults, immunocompromised and immunocompetent patients, and different sampling sites, primers, PCR probes and definitions for pneumococcal CAP[10-13]. In both children and immunocompromised individuals pneumococcal colonization density is likely higher, resulting in different optimal cut-off values[14].

To date the optimal sampling site remains unknown, which could explain the discrepancies between in-vitro and in-vivo performance. Some studies use sputum samples whereas others use nasopharyngeal swabs or oropharyngeal (OP) swabs[4,9,12,13]. Three studies comparing rt-qPCR lytA in sputum versus nasoparhyngeal swabs had conflicting results with 2 finding higher sensitivity in sputum samples and the third showing a comparable diagnostic performance[4,12,13].

One study compared trans-nasal and trans-oral sampling and concluded that the nasopharynx is the main reservoir for *S. pneumoniae*, but data on the best sampling technique is limited and it is unclear which is superior[15]. According to the WHO Pneumococcal Carriage Working Group NP samples have a slightly higher sensitivity in detecting colonization with *S. pneumoniae* in healthy adults and children. A combination of NP and OP samples is recommended for detection of *S. pneumoniae* carriage in adults[16,17]. However, there are no current recommendations about molecular diagnostics and detection of *S. pneumoniae* in patients with CAP.

Previously the usefulness of the qPCR has been questioned in patients who were pre-treated with antibiotics[3,18]. The total number of patients who have been pre-treated

with antibiotics in our validation study is rather low (16.1%), precluding our capability to draw firm conclusions.
Overall, we were able to validate a rt-qPCR for *lytA* with good in-vitro test characteristics and promising in-vivo results.
Future studies should focus on determining the best sampling site, establishing optimal cut-off values distinguishing colonization from infection for different patients groups, quantify the effect of pre-treatment with antibiotics and increasing specificity of the *lytA* qPCR by refining primers and probes used in the qPCR to diminish cross-reactivity between similar species to *S. pneumoniae*. Since publication of our study we have refined our *own lytA* PCR to have less overlap with other streptococci[19].
Finally, when the above questions have been answered, use of the qPCR should be prospectively validated in an unselected cohort of CAP patients and to determine the additional value of the qPCR in clinical practice through better microbiological diagnostics guiding antibiotic regimes. Now, this is investigated in our hospital in a joint venture with Streeklab Haarlem and RIVM.

Diagnosing Legionella pneumonia

CAP caused by *Legionella* has a high mortality rate and incidence is increasing[20-23].
It requires targeted antibiotic treatment, in an era where antibiotic resistance is rising and antibiotic stewardship is important. Although clinical symptoms of *Legionella* prove non-specific, they are often used in daily clinical practice as a decisive factor in the empiric antibiotic regime[24-27].
In **chapter 3** we validated a *Legionella* prediction score based on six clinical parameters that can easily be obtained upon admission. The score had a high accuracy with an AUC of 0.89 (95% CI 0.86–0.93) and could be potentially useful to rule-in or rule-out *Legionella* CAP, depending on the cut-off point chosen. A score of ≥4 to rule-in *Legionella* resulted in a specificity of 93.1% with a sensitivity of 58.8%.
Therefore, the score holds promise for early identification and specific treatment of those infected with *Legionella*, in particular in cases not detected by UAT, which only tests for *Legionella pneumophila* serogroup 1. Up to 20-50% of *Legionella* cases worldwide are estimated to be caused by different *Legionella pneumophila* serogroups or different *Legionella* species[28-30].
The negative predictive value of the score will likely be higher in an unselected population of hospital admitted CAP patients, since the incidence of *Legionella* is lower than in our population.
All predictors were associated with the outcome. However, temperature and platelets were no longer significantly associated after multivariate analysis and dichotomization, which might be explained by the wide range in which these variables occurred in both patients with *Legionella* CAP and with non-*Legionella* CAP.
Our study yielded an accuracy similar to that found in a Spanish study (AUC 0.86 (95% CI 0.81–0.90)), based on 82 *Legionella* cases[31]. In another multinational validation study with a sample size of 37 *Legionella* cases accuracy was lower with an AUC of 0.73 (95% CI 0.65–0.81)[32]. Baseline differences between cases and controls with respect to age, COPD and smoking status in our study were similar to both other studies. In a Japanese validation study with higher proportion of males and also including patients with cancer, sensitivity was 94% and a specificity 49% at a cut-off ≥2, resembling results of our study[33]. The same study group proposed a variation of the score which included gender and dyspnoea instead of temperature and platelets, which performed well in their validation cohort (AUC 0.93). However, in populations outside of Japan male gender and dyspnoea were not identified as risk factors for *Legionella*-related CAP[34].
Two other diagnostic scoring systems for *Legionella*-related CAP have been proposed, namely the Winthrop University score and the Community-Based Pneumonia Incidence Study Group scoring system. Both scoring systems were validated, but appeared unsuitable for diagnosing or excluding *Legionella* in a clinical setting, due to low accuracy and/or the need to include follow-up data[33,35,36].
To date our validation study included the largest number of patients (n=131) with *Legionella*-related CAP, and with sufficient number of participants required for validation of a prediction score with a dichotomous outcome[37].
All hospital admitted patients with CAP were eligible for inclusion and data was collected from five different large hospitals with a wide geographical spread. This adds to the external validity of the study because it closely resembles a real-life clinical population.
However, a notable study limitation is the retrospective design which led to many exclusions due to missing data especially for the variable "dry cough". In a prospective study, this parameter would be easy to obtain. We chose not to impute missing data which adds to the internal validity of the study, but this has the potential to introduce selection bias. Given the large sample of patients the effect of this potential bias is likely small.
Furthermore, cases were retrospectively selected, based on positive microbiological tests, mostly UAT. As this test only detects *Legionella pneumophila* serogroup 1, and since cultures and PCR have not been performed in all participants, some episodes of Legionella infection might have been missed. This could have influenced the performance of the score.
In a Japanese study the *Legionella* prediction score had better performance for *Legionella* serogroup 1 (N=11) than for other *Legionella* species (n=23)[38]. This suggests that the score is particularly useful for detecting *Legionella* serogroup 1, which was detected in 96% of the cases in the present study.
Future studies should aim to validate the diagnostic score prospectively, preferably in an unselected CAP population where extensive testing for *Legionella* is routinely employed with urinary antigen tests, PCR and cultures. Ideally future studies should focus on the accuracy of the scoring system in different *Legionella* serogroups or species, give more insight into performance of the score over the course of the disease, mild versus advanced disease, and investigate its clinical significance in addition to UAT. Moreover, longitudinal studies on clinical outcomes resulting from implementation of the score, such as change in antibiotic prescriptions, mortality, ICU admissions and of length of stay, are needed.

Cytokines and aetiology of CAP

In **chapter 4** the differences in systemic cytokine levels between several well-defined aetiologies of CAP have been discussed. There were differences for IL-6, IL-10, IL-17A and IFN-γ, yet with considerable overlap between groups, and 2 models were created:

one based on cytokines alone and one incorporating CRP levels to distinguish viral from bacterial CAP. The models performed reasonably well with AUCs of 0.86 and 0.91, respectively, with the best model able to achieve a sensitivity of 18% and specificity of 99% for viral CAP given a cut-off point AUC of 0.65. However, our study is merely a proof of concept due to some important limitations.
Previous studies have focused on individual biomarkers to differentiate aetiology in CAP. The best-studied biomarker is the plasma IL-6 level. IL-6 was significantly different between typical and viral CAP, between pneumococcal CAP and *Mycoplasma pneumoniae* and between pneumococcal and non-pneumococcal CAP respectively[39-41].
Yet, CAP severity scores and short term mortality also correlate with cytokine IL-6 levels on the first day of hospitalization[42,43]. This poses the question whether cytokine levels are specific for aetiology, specific for disease severity, or both. Menendez et al. demonstrated that peripheral IL-6 is elevated in CAP presenting with acute sepsis or shock despite aetiology, but is also elevated in CAP caused by Gram-positive cocci without septic shock[44]. Endeman et al. reported that IL-6 levels in blood were higher in pneumococcal CAP independent of age and PSI[39].
A limitation of the study described in chapter 3 is the retrospective design (although the data and samples were prospectively collected), with selected patient groups and excluding patients in which no pathogen was detected or admitted in ICU. We used a PCT cut-off of <0.25 µg/L to define a strict-viral group in which we deemed presence of an undetected bacterial co-infection highly unlikely. Indeed when we performed a sensitivity analysis where we excluded PCT as a selection criterion the model performed worse.
Choosing a PCT criterion reduces, but not completely rules out, the likelihood of including individuals with undetected bacterial pathogens to our strict viral CAP group. PCT levels were found to differentiate typical from atypical CAP, but not atypical from viral CAP[45,46]. So, atypical pathogens that were not detected by PCR, could potentially have been included in our presumably strict viral group.
Exclusion of patients with an indefinite microbial cause will create considerable bias. Other studies reported higher IL-6 levels in the known-aetiology group compared to the unknown-aetiology group[44]. Only patients with a definite microbial diagnosis were selected for our model, and in general a definite microbiological diagnosis is established in only 40%-50% of patients presenting with CAP[47-50]. Furthermore, our group definitions may influence results. We excluded adenoviruses and rhinoviruses - when present as only pathogen - as causative pathogen[47,51].
We chose the pneumococcal CAP group for its homogeneity, but did not select other bacterial CAP groups, because these groups would be too small for meaningful statistical analysis. We expected the mixed CAP group to be heterogeneous, both in aetiology as in cytokine expression. Subgroup analysis was not meaningful with a maximum of nine subjects having comparable co-infection (influenza-pneumococci). Noteworthy, is the absence of atypical pathogens in our mixed CAP group, despite the use of routine PCR on oropharyngeal swab, which increases detection rate.
Furthermore, we did not take pretreatment with antibiotics or prednisone prior to admission into account. In previous studies antibiotic pre-treated patients appeared to have lower IL-10 and IL-6 levels, compared to treatment-naïve patients[39,41,44]. Corticosteroids also influence production of, for example, IL-6, IL-8 and TNF-α. However, this effect was greater in patients with atypical CAP compared to pneumococcal CAP

where it barely influenced cytokine levels[52].
There also remains uncertainty about the effect of COPD on our results, since chronic lung inflammation in COPD patients may alter the immune response towards a Th-1 direction[53,54]. In COPD patients with CAP causality between pathogen detection and disease is challenging. For instance, potential pathogens can be detected in the lower airways of 25% of ambulant COPD patients while not suffering from an exacerbation, and in 52% of those with exacerbation[55].
Finally, timing of measurement may have influenced our results. Cytokine levels generally decline in the course of disease, influence other pathways or alter after treatment is initiated[39,56].
Future research should first focus on the effects of relevant factors that influence cytokine expression, such as pre-treatment with antibiotics or concurrent treatment with corticosteroids, concomitant COPD, timing of measurement, severity of disease and relation with specific pathogens. Subsequently, it should focus on distinguishing between true viral, mixed and other bacterial infections, as this is clinically most relevant. When a viable cytokine expression pattern is found it should be validated prospectively in an independent cohort of CAP patients with a focus on the costs and benefits of implementation of such a strategy to reduce unnecessary antibiotic use.

CRP guided and PCT guided antibiotic treatment

In **chapter 5** we have reported on the effects of two feedback strategies - CRP-based and PCT-based algorithms - for discontinuation of antibiotic treatment during a 30 day follow-up period, compared to standard care, in patients hospitalized with CAP in non-ICU wards. In this study we aimed to mimic routine clinical practice with broad inclusion criteria, only excluding groups that would not be treated as regular CAP based on national guidelines, using a pragmatic approach to counting antibiotic treatment in days and taking blood samples during regular laboratory rounds. Previous studies have suggested that antibiotic duration can be shortened based on clinical parameters, such as the criteria for clinical stability defined in the IDSA guidelines[27,57,58]. However, some patients do not reach these criteria, even at the time of discharge. In our study this applied to 10.6% of the patients.
In both biomarker groups 93 (43.9%) and 47 (15.1%) patients were treated for 3 and 4 days, respectively. Overall, 45% of the patients in the intervention groups received antibiotic treatment for less than 5 days, which reflects a considerable reduction in antibiotic consumption.
Furthermore, despite evidence that shorter antibiotic courses are safe, most physicians still treat patients hospitalized with CAP for 7-10 days and even longer[59,60]. Although Dutch guidelines have recommended antibiotic courses of 5 days for patients with CAP with good clinical recovery at day 3 since 2011, only 18 of 156 (11.5%) patients in the standard care treatment group were treated for less than 7 days in the current study. In another more recently performed Dutch multi-centre study focusing on antibiotic stewardship addressing a similar patient population the average duration of antibiotic treatment was 6.6 days[2]. Antibiotic use is an important driver of antimicrobial resistance, and shortening of treatment duration reduces antibiotic selective pressure. This underlines the need for simple and objective criteria to change clinical practice.

In our study both CRP-based and PCT-based algorithms reduced the median days of antibiotic use in the first 30 days after admission from 7 to 4 and 5.5, respectively. A CRP based algorithm has advantages over a PCT based algorithm. CRP is a widely used, cheap (er) and readily available biomarker in nearly every clinical setting. Point-of-care CRP testing was effective in reducing antibiotic consumption for lower respiratory tract infections in nursing homes[61].

Yet, there is little evidence to support CRP measurements to tailor the duration of antibiotic treatment in patients with CAP. In one study failure of CRP to decline within the first few days of hospitalization was associated with a poor prognosis of CAP[62]. Only once has a CRP based algorithm been compared to a PCT based algorithm[63]. In that trial of 94 ICU-patients with sepsis, 49 were allocated to PCT and 45 to CRP measurements, without a control group. Median duration of treatment was 6 days in the CRP group and 7 days in the PCT group, with comparable outcomes between groups. The same group studied a modified version of their CRP algorithm in an open label RCT in ICU patients and found a small reduction in antibiotic treatment time in favour of the CRP group[64]. However, their algorithm differed from ours in that CRP had to decline with 50% in patients with an initial CRP >100 mg/L and to <35 mg/L in patients with an initial value <100 mg/L.

Our PCT-based algorithm reduced antibiotic exposure but resulted in slightly more new antibiotic prescriptions during follow-up compared to the control group. This might have resulted, in part, from the fact that less patients in the PCT group received empirical therapy that covered atypical pathogens than the other study groups (table 1). Several other large, well-designed trials and a Cochrane review have been conducted in a variety of clinical settings, but none of those reported more treatment failure in the PCT groups[65,66].

In a Swiss study a PCT based algorithm reduced the duration of antibiotic treatment to a median of 5 days in patients with radiologically proven CAP with similar rates of antibiotic prescription in long term follow-up. However, their algorithm allowed clinicians more freedom and their definition of treatment failure was limited to symptoms related to CAP. Lastly, combination therapy was started in 34% of patients, as compared to 24% of patients in our study[67].

Despite all evidence addressing PCT, there are still concerns about exclusion rates in clinical trials, uncertainty with regards to the necessity of overruling of the PCT algorithm in trials by treating physicians and the usefulness of PCT in patients with atypical pathogens, COVID-19, renal failure or critically ill patients[68-72].

Our study has several limitations. First, our study was underpowered to exclude harm due to reduced duration of antibiotic treatment, as recently recommended by the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) working group on design of antimicrobial stewardship evaluations[73]. Ideally such a trial would include all patients admitted with CAP and should demonstrate superiority in reducing antibiotic exposure over standard clinical practice and simultaneously demonstrate non-inferiority for unwanted clinical outcomes. Short antibiotic courses carry the risk of undertreatment, leading to recurrence or worsening of symptoms, additional antibiotic prescriptions, and increased time to recovery. Based on a meta-analysis of 26 RCT's there is no evidence that PCT based treatment strategies carry any of these risks[74].

In the current study 41 of 312 patients received a new antibiotic prescription after a short initial course based on the biomarker algorithms, and 23 of these prescriptions occurred within a week after antibiotic treatment was stopped. It is unlikely that all of these could have been prevented with a longer initial course, since some of these were due to culture results yielding pathogens resistant to empiric therapy.

The potential harm of short antibiotic treatment should be weighed against the harms of excess antibiotic treatment. Excess antibiotic treatment does not seem associated with lower rates of adverse outcomes, including death, readmission and emergency department visits[75].

Second, CRP measurements were frequently used in our control group and to a lesser extent in our PCT group. It is unclear if these measurements influenced clinical decision making and study outcomes. If they did it would most likely lead to an underestimation of the effect of our CRP algorithm. In our PCT group CRP measurements did not influence antibiotic treatment duration. Even if they did, it would lead to an overestimation of the effect of the PCT algorithm.

Third, the observed 30-day mortality rate in our study is relatively low (1.9%), even though 15% of our patients classified as severe pneumonia (≥3) according to the CURB-65 score. Reported 30-day mortality rates for hospitalized non-ICU patients with CAP range from 5-10%. The low mortality could have resulted from the study design, in which patients had to decide on day 2 or 3 on study participation, which may have selected for a less sicker study population. For instance, informed consent on admission was not possible e.g. due to delirium in 53 patients, which could, therefore, not be included. Overall, 450 out of 1434 screened patients were eligible for the intervention but were not included or randomized, compared to 468 randomized patients. This limits the generalizability of our findings. Fourth, post-discharge sampling to determine biomarkers was part of study protocol, but may not be realistic in routine daily care. In 62 of 88 patients in whom blood samples were taken at home or on outpatient visits, antibiotics were discontinued because of biomarker measurements. This also limits the generalizability of our results. Fifth, the feedback of biomarker results to treating physicians was an important part of the intervention tested, implying that the effectiveness of the intervention may well be less when implemented without active feedback. Lastly, there is a considerable publication delay. However, the research question regarding biomarker based strategies is still relevant today, and the average treatment durations in our control group are comparable to those reported in similar patient populations in recently published studies[2,57].

In conclusion, in this study both CRP and PCT based treatment algorithms reduced the duration of antibiotic treatment in patients admitted to a regular hospital ward with CAP. Future studies should focus on the non-inferiority of this approach with respect to clinically relevant patient centered outcomes.

MR-proADM

In **chapter 6** we have performed a matched case control study and MR-proADM levels appeared comparable between CAP patients with and without a negative outcome. These findings do not support the usefulness of MR-proADM as a prognostic variable in patients hospitalized with CAP.

Most prior studies have focused on the value of MR-proADM as a substitute for or an

enhancement to existing risk scores. Our findings did confirm results of several other studies that MR-proADM levels were associated with CURB-65 scores[69,76-78].
Moreover, in several studies and a meta-analysis elevated MR-proADM levels were associated with a higher risk of mortality and cardiovascular events[77-79].
Since we studied patients admitted to non-ICU wards, mortality in our population was low precluding meaningful comparisons with MR-proADM. Moreover, among patients presenting to the emergency department with lower respiratory tract infections Albrich et al. found a significant association between biomarkers and ICU-admission and empyema[76]. Similarly, Bello et al. found an association between a wide variety of possible complications that included cardiac failure, renal failure, septic shock and new hyperglycaemia as well as pulmonary complications such as empyema, pleural effusion or respiratory failure with or without acidosis[77].
A third study reported a statistically significant correlation between MR-proADM and respiratory failure/shock and need of ICU admission in patients with CAP[80].
However, less is known about associations between MR-proADM levels and treatment outcomes in patients hospitalized in non-ICU wards. Multiple studies have been performed with multiple endpoints, yielding different results[81-83].
Our study's matched case-cohort design does come with some inherent limitations regarding variability and generalizability to the broader CAP population, especially since our sample size is relatively small, excludes ICU-patients and 30-day mortality is relatively low. Furthermore, the design inherently precludes definitive statements regarding the predictive value of MR-proADM in this context.
However, it possesses a notable strength: we conducted matching within the same CAP population, distinguishing our approach from other studies that paired pneumonia patients with individuals admitted to different hospital wards. As a result, it enhances the comparability of our cases and controls, providing a clearer framework for evaluating differences in MR-proADM and negative outcomes. Since all outcomes were prospectively recorded the risk of attention or recall bias is lower than if outcomes were retrospectively assessed.
Overall our results do not support MR-proADM as a prognostic variable once the patient has been hospitalized. Future studies should primarily focus their efforts on developing prospective clinical algorithms to determine if MR-proADM could or should impact the treatment of admitted patients.

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Chapter 8

Summary

ENGLISH SUMMARY

In **chapter 2** the development of a real-time quantitative polymerase chain reaction (rt-qPCR) targeting the *lytA* gene present in pneumococci was described. The aim of this study was to validate this PCR in vitro with different strains of *Streptococcus* species and in vivo using oropharyngeal swabs from hospitalized patients with CAP. Intra- and inter-run variability, in vitro specificity and sensitivity, and the lower limit of detection were determined. In addition, a pilot-study was performed with samples from 28 patients with pneumococcal pneumonia and 28 patients with pneumonia without detection of *S. pneumoniae* (but with detection of either a viral and or another bacterial pathogen) to validate this test. Intra- and inter-run variability were relatively low (SD's ranging from 0.08 to 0.96 cycle thresholds) and the lower limit of detection was 1-10 DNA copies/reaction. In-vitro sensitivity and specificity of the tested specimens (8 strains carrying lytA and 6 strains negative for *lytA*) were both 100%. In patients with pneumococcal and non-pneumococcal pneumonia a cut-off value of 6.000 copies/ml yielded sensitivity of 57.1% and specificity of 85.7%. Overall, the rt-qPCR for *lytA* had good in-vitro test characteristics and promising in-vivo results.

In **chapter 3** an existing *Legionella* prediction score to detect CAP caused by *Legionella* on admission was validated. The score consists of 6 items easily obtainable on admission, each yielding one point if present: fever >39.4°C; dry cough; hyponatremia (Na) <133 mmol/L; lactate dehydrogenase (LDH) >225 mmol/L; C-reactive protein (CRP) >187 mg/L and platelet count <171 x 109/L. Patients with *Legionella*-related CAP admitted to five large Dutch hospitals between 2006 and 2016 (n=131) were included, as were 160 non-*Legionella*-related CAP patients. The accuracy of the prediction score was assessed by calculating the area under the curve (AUC) through logistic regression analysis. A score of 0 occurred in non-*Legionella*-related CAP patients only, a score of 5 and 6 in *Legionella*-related CAP patients only. A cut-off ≥4 resulted in sensitivity of 58.8% and specificity of 93.1% with an AUC of 0.89 (95%CI 0.86-0.93). The strongest predictors for CAP caused by *Legionella* were elevated LDH, elevated CRP and hyponatremia. To summarize, the *Legionella* prediction score had high diagnostic accuracy in a large group of patients shows promise for future prospective validation to better target antibiotic treatment for suspected *Legionella* CAP.

In **chapter 4** it was hypothesized that cytokine expression (IL-6, IL-10, IL-27, IFN-γ and CRP) on admission in CAP patients differs between causative pathogens, and the aim was to develop a cytokine based prediction model to identify episodes with a strict viral aetiology. Plasma cytokine patterns were determined in a cohort of pneumonia patients retrospectively categorized as strict viral, pneumococcal or combined viral and bacterial CAP. Among 344 CAP patients 159 had a detectable pathogen. Patients with adenovirus or rhinovirus as sole pathogens were excluded, as were patients in the strict viral group with a procalcitonin >0.25µg/L. Overall, 104 patients were categorized as either strict viral CAP (n=17), pneumococcal CAP (n=48) or bacterial/viral CAP (n=39). Strict viral CAP was predicted by logistic regression using multiple cytokine levels (IL-6, IL-27 and CRP) with an AUC of 0.911 (95% CI: 0.852 – 0.971, p<0.001). For the same patients the AUC of CRP was 0.813 (95%-CI: 0.728 – 0.898, p<0.001). In conclusion, there were discernible aetiology-related differences in cytokine expression in selected CAP patients. Prospective validation studies are warranted.

In **chapter 5** the effectiveness of active feedback of treatment algorithms based on procalcitonin (PCT) and C-reactive protein (CRP), compared to standard care, on the duration of antibiotic treatment in patients hospitalized with community-acquired pneumonia (CAP) in non-ICU wards was assessed in a randomised, open label, parallel group, multi-centre trial in 3 Dutch teaching hospitals. Treatment duration was guided by a PCT algorithm, CRP algorithm or standard care. Participants were recruited by a member of the study team and randomised at day 2-3 of admission in a 1:1:1 ratio. Treatment was discontinued upon predefined thresholds of PCT or CRP that were assessed on admission, day 4 and days 5-7 if indicated. The primary outcome was total days on antibiotic treatment until day 30.
In total 468 participants were randomized. The median days on antibiotics (IQR) was 7 (IQR 7-10) in the control group, 4 (IQR 3-7) in the CRP group (rate ratio (RR) of 0.70, 95% CI 0.61 - 0.82 compared to standard care; p <0.001), and 5.5 (IQR 3-9) in the PCT group (RR of 0.78, 95% CI 0.68 - 0.89 compared to standard care; p <0.001). New antibiotics within the first 30 days were prescribed to 24, 23 and 35 patients in standard care, CRP and PCT groups, respectively. The hazard ratio for a new prescription in patients in the PCT group compared to standard care 1.63 (CI 0.97 - 2.75; p = 0.06). There were no statistically significant differences in time to clinical stability or length of stay. IN summary, a strategy of feedback of CRP-guided and PCT-guided treatment algorithms reduced the number of days on antibiotic in the first 30 days after hospital admission in non-ICU wards for CAP. The study was underpowered for evaluating safety of shortening duration of antibiotic treatment.

In **chapter 6** levels in MR-proADM at the time of admission in patients admitted with CAP to a non-ICU hospital ward were determined in 45 matched case-control pairs, and related to short-term adverse outcomes, including treatment failure, short-term mortality and re-admission after discharge. MR-proADM levels were comparable between patients developing and not developing adverse outcomes. MR-proADM values increased with higher CURB-65 score (p < 0.001). These findings do not support the use of MR-proADM as a prognostic variable in patients hospitalized with CAP.

Chapter 9

Nederlandse samenvatting
(Dutch summary)

Hoofdstuk 2 beschrijft de ontwikkeling van een real-time kwantitatieve polymerasekettingreactie (rt-qPCR) gericht op het *lytA*-gen aanwezig in de celwand van pneumokokken. Het doel van deze studie was om de PCR in vitro te valideren met verschillende beschikbare stammen van streptokokkensoorten en in vivo met behulp van orofaryngeale uitstrijkjes van klinisch opgenomen patiënten met longontsteking. We testten eerst de intra- en inter-run variabiliteit. Ook werd de in vitro specificiteit en sensitiviteit, inclusief de ondergrens van detectie, bepaald. Daarnaast werd een pilotstudie uitgevoerd met monsters van patiënten (n=28) met pneumokokkenpneumonie en patiënten (n=28) met een pneumonie met een andere verwekker, dat wil zeggen een virale en/of een andere bacteriële infectie. De intra- en inter-run variabiliteit waren relatief laag (standaarddeviaties variërend van 0,08 tot 0,96 cycle thresholds). De ondergrens van detectie bleek 1-10 DNA-kopieën/reactie te zijn. De in vitro gevoeligheid en specificiteit van de geteste monsters (8 stammen met *lytA* en 6 stammen negatief voor *lytA*) waren beide 100%. In de klinische samples met pneumokokken- en niet-pneumokokken-pneumonieën zou een afkapwaarde van 6.000 kopieën/ml leiden tot een gevoeligheid van 57,1% en een specificiteit van 85,7%. Over het algemeen konden we de rt-qPCR voor *lytA* valideren met goede in vitro testkenmerken en veelbelovende in vivo resultaten.

Hoofdstuk 3 beschrijft het valideren van een bestaande *Legionella*-voorspellingsscore met de bedoeling om *Legionella*-gerelateerde CAP bij opname te detecteren. De score bestaat uit 6 items die makkelijk te verkrijgen zijn bij opname en elk positief item wordt beloond met één punt: koorts >39,4°C; droge hoest; hyponatriëmie (Na) <133 mmol/L; lactaatdehydrogenase (LDH) >225 mmol/L; C-reactief proteïne (CRP) >187 mg/L en trombocytenaantal <171 x 10^9/L. Patiënten met *Legionella*-gerelateerde CAP opgenomen in vijf grote Nederlandse ziekenhuizen tussen 2006 en 2016 werden geïncludeerd. Controles waren niet-*Legionella*-gerelateerde CAP-patiënten. De nauwkeurigheid van de voorspellingsscore werd beoordeeld door het berekenen van het gebied onder de curve (AUC) via logistische regressieanalyse. We includeerden 131 patiënten met *Legionella*-gerelateerde CAP en 160 controlepatiënten. Een score van 0 kwam alleen voor bij niet-*Legionella*-gerelateerde CAP-patiënten, een score van 5 of 6 alleen bij *Legionella*-gerelateerde CAP-patiënten. Een afkapwaarde ≥4 resulteerde in een gevoeligheid van 58,8% en een specificiteit van 93,1%. De AUC was 0,89 (95%CI 0,86-0,93). De sterkste voorspellers waren LDH, CRP en laag natrium. Over het algemeen konden we de *Legionella*-voorspellingsscore valideren in een grote groep patiënten, die een hoge diagnostische nauwkeurigheid liet zien. De score moet in de toekomst prospectief gevalideerd worden en zou kunnen bijdragen aan gerichte antibioticabehandeling van vermoedelijke *Legionella*-CAP.

In **Hoofdstuk 4** is de hypothese geformuleerd dat de expressie van cytokines bij opname van CAP-patiënten varieert afhankelijk van de veroorzakende pathogenen. Het doel was om een voorspellend model op basis van cytokines te ontwikkelen, dat in staat is een strikt virale etiologie nauwkeurig te voorspellen, aangezien deze onderscheiding klinisch het meest relevant is. De plasma-cytokinepatronen zijn geanalyseerd bij retrospectief gecategoriseerde groepen op basis van etiologie: strikt virale, pneumokokken- of gecombineerde virale en bacteriële CAP. Van de 344 CAP-patiënten hadden 159 een aantoonbaar pathogeen. Patiënten met adeno-/rhinovirus infecties werden uitgesloten.

Eveneens werden patiënten in de strikte virale groep met een procalcitoninegehalte (PCT) van meer dan 0,25 µg/L uitgesloten. Uiteindelijk werden 104 patiënten in 3 groepen ingedeeld: strikt virale CAP (n=17), pneumokokken-CAP (n=48) en bacteriële/virale CAP (n=39). Bij alle patienten werden de cytokines IL-6, IL-10, IL-27, IFN-γ en CRP gemeten. Een logistiek regressiemodel dat gebruik maakt van meerdere cytokines (IL-6, IL-27 en CRP) behaalde een Area Under the Curve (AUC) van 0,911 (95% CI: 0,852 - 0,971, p<0,001) voor het voorspellen van strikt virale CAP-etiologie. Ter vergelijking was de AUC voor CRP bij dezelfde patiënten 0,813 (95% CI: 0,728 - 0,898, p<0,001). Concluderend benadrukken deze bevindingen onderscheidbare verschillen in cytokine expressie gerelateerd aan etiologie bij een subset van CAP-patiënten. Prospectieve validatiestudies zijn noodzakelijk om deze waarnemingen te bevestigen.

In **hoofdstuk 5** is gekeken naar de effectiviteit van actieve terugkoppeling van behandelalgoritmes gebaseerd op procalcitonine (PCT) en C-reactief proteïne (CRP), vergeleken met standaardzorg, met betrekking tot de duur van antibiotische behandeling bij patiënten opgenomen in het ziekenhuis met community-acquired pneumonia (CAP) op niet-IC-afdelingen. Dit werd gedaan middels een gerandomiseerde, open-label, parallelle groep, multicentrische studie in 3 grote Nederlandse ziekenhuizen. De behandeling werd geleid door een PCT-algoritme, CRP-algoritme of standaardzorg. Deelnemers werden geworven door een lid van het onderzoeksteam en gerandomiseerd op dag 2-3 van opname in een verhouding van 1:1:1. De antibiotica behandeling werd stopgezet bij vooraf gedefinieerde drempels van biomarkers die werden beoordeeld bij opname, dag 4 en dagen 5-7 indien nodig. Het primaire eindpunt was het totaal aantal dagen antibiotische behandeling tot dag 30. In totaal werden 468 deelnemers geïncludeerd in deze studie. De mediane dagen antibiotica waren 7 (IQR 7-10) in de controlegroep, 4 (IQR 3-7) in de CRP-groep (rate ratio (RR) van 0.70, 95% CI 0.61 - 0.82 vergeleken met standaardzorg; p <0.001), en 5,5 (IQR 3-9) in de PCT-groep (RR van 0.78, 95% CI 0.68 - 0.89 vergeleken met standaardzorg; p <0.001). Nieuwe antibiotica werden voorgeschreven aan respectievelijk 24, 23 en 35 patiënten in de standaardzorg, CRP- en PCT-groepen binnen de eerste 30 dagen. Het risico op een nieuw voorschrift bij patiënten in de PCT-groep vergeleken met standaardzorg was 1.63 (CI 0.97 - 2.75; p = 0.06). Er werd geen verschil gevonden in de tijd tot klinische stabiliteit of de duur van opname. Over het geheel genomen verminderde een strategie van feedback van CRP-geleide en PCT-geleide behandelalgoritmes het aantal dagen antibiotica in de eerste 30 dagen na ziekenhuisopname op niet-IC-afdelingen voor CAP. De studie had te weinig deelnemers om ook de veiligheid van verkorting van de duur van antibiotische behandeling te bepalen.

In **hoofdstuk 6** zijn verschillen in MR-proADM-niveaus bij opname bepaald tussen gematchte casus en controles, met de focus op nadelige uitkomsten op korte termijn, waaronder falen van behandeling, korte-termijn mortaliteit en heropname na ontslag bij patiënten opgenomen met CAP op een niet-IC-ziekenhuisafdeling. In totaal werden 45 cases gematcht aan 45 controles. MR-proADM-waarden waren vergelijkbaar tussen patiënten die negatieve uitkomsten ontwikkelden en degenen die dat niet deden. MR-proADM-waarden namen toe met een stijgende CURB-65-score, met p < 0,001. Deze bevindingen ondersteunen het nut van MR-proADM als een prognostische variabele bij patiënten opgenomen met CAP niet.

Appendices

Ruud Duijkers was born september 1, 1987 in Zaanstad, the Netherlands. He studied medicine from 2005 to 2012 at the 'Vrije Universiteit' in Amsterdam. In 2012 he started working as a medical doctor at the pulmonology department in the Northwest Hospital group in Alkmaar. After a year he started his PhD project titled *"Reduction of Antibiotic Therapy by Biomarkers in Patients With CAP Episodes (REDUCE Study)"* which was a multicenter trial in 3 Dutch hospitals. The results can be found in chapter 4. During this time he developed and worked on several other projects which would form the basis of the other chapters in this thesis. As a side project he was closely involved in several COPD studies, most notably the CATCH study which treated patients presenting with an acute exacerbation of COPD based on CRP levels and compared the outcomes to regular clinical care. In 2017 he moved to Leeuwarden to start his residency in Pulmonology. During his residency, his dedication to pulmonary infectious diseases grew stronger. Towards the end of his training, he played a key role in managing the outpatient clinic tailored specifically for patients with pulmonary infectious diseases at an academic tertiary referral center.

Ruud lives together with his wife Ellen in Leeuwarden and hopes to finish his residency by 2025.

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Dankwoord

Zoveel mensen hebben de afgelopen 10 jaar bijgedragen aan het tot stand komen van dit proefschrift dat het onmogelijk is om iedereen individueel te bedanken. Daarom ook iedereen die hieronder niet in het bijzonder wordt genoemd, hartelijk dank voor jullie bijdrage!
Ook wil ik alle proefpersonen bedanken die hun medewerking hebben verleend aan het onderzoek. Ik heb veel van u allen mogen leren en een groot deel van u thuis mogen bezoeken voor de bloedafnames. Dit heeft een unieke inkijk gegeven in het herstel na ontslag uit het ziekenhuis en is zeer waardevol, niet alleen als onderzoeker, maar vooral ook als dokter. Zonder u allen was dit onderzoek niet mogelijk geweest.

Beste Wim, uiteraard moet ik jou als eerste bedanken. Dank voor de mogelijkheden en alle vrijheid die je me geboden hebt in het bedenken, opzetten en uitvoeren van de onderzoeken in dit proefschrift. Je stond altijd paraat om advies en wijze raad te geven waar dat nodig was. Er zijn vele momenten geweest dat de moed mij in de schoenen zakte, maar toch wist je me altijd in korte tijd te enthousiasmeren om door te gaan.
Ook al moet je zelf soms ook met je handen in het haar gezeten hebben in tijden dat het onderzoek stil lag en dingen niet gingen zoals we graag hadden gezien. Desondanks leek je er altijd vertrouwen in te hebben dat we het samen tot een goed einde konden brengen. Zonder jouw begeleiding denk ik niet dat dit proefschrift ooit het licht had gezien, daarvoor ontzettend bedankt!

Uiteraard ook mijn promotor professor Bonten heel erg bedankt. Ik heb veel mogen leren van de waardevolle feedback over de jaren. Zeker in het begin met alle drukte van het onderzoek is niet alles gladjes verlopen, maar wrijving brengt uiteindelijk glans. Dank voor het geduld, de geboden mogelijkheden en de kritische blik; niet alleen het proefschrift is daar beter van geworden maar ik ben er van overtuigd dat het me ook in de dagelijkse praktijk als dokter veel gebracht heeft in de manier van kijken naar onderzoek en dit vertalen naar de kliniek.

Beste Henri, ook jou wil ik ontzettend bedanken voor de waardevolle hulp bij het uitwerken van het onderzoek. Ik heb veel bewondering voor jouw geduld, vriendelijkheid en de manier waarop je ingewikkelde dingen begrijpelijk kunt maken.

Dominic Snijders bedankt voor het mede bedenken van dit promotieonderzoek en de begeleiding in het begin. Door jou had ik enig idee waar ik aan begon en wat een promotietraject met zich mee brengt. Ook dank voor de tomeloze inzet om van het Slotervaart ziekenhuis een deelnemend centrum te maken!
Martijn Kross en Jan Willem van den Berg, dank voor jullie deelname en inzet aan het onderzoek. De extra centra waren hard nodig om de inclusie van het onderzoek tijdig af te krijgen en jullie inzet bij en voor de patiënten was essentieel om dit tot een goed einde te brengen.

Alle (oud) arts-assistenten van Alkmaar, Zwolle en het Slotervaart ontzettend bedankt voor jullie inzet, wat zullen jullie af en toe hebben moeten zuchten als er midden in

de nacht weer een geschikte studiepatiënt naar de spoed kwam die mee wilde doen aan het onderzoek. Gelukkig hebben jullie je altijd in willen zetten voor het onderzoek en hebben velen van jullie niet geschroomd om toch ook tijdens drukke avond-/nachtdiensten de telefoon te pakken en te bellen als er iets niet duidelijk was. Dit is van onschatbare waarde geweest voor de snelheid van de inclusie. Zonder jullie was de studie niet mogelijk geweest!

Beste Anke, Lida, Laura en Eva. Jullie zijn de reden dat ik af en toe het onderzoek in goede handen achter durfde te laten. Jullie zijn ook geregeld als het nodig was voor mij op pad gegaan in het weekend om bij patiënten thuis de bloedafnames te doen. Jullie zijn van onschatbare waarde geweest in de uitvoering van de REDUCE. Ook dank voor alle gezelligheid op de werkvloer!

Ook dank aan alle verpleegkundigen en secretaresses die hun bijdrage hebben geleverd aan het onderzoek. Jullie hebben veel praktische dingen moeten regelen qua bloedafnames, planning van afspraken en zoveel andere dingen meer. Toch waren jullie altijd bereid om alles te regelen alsof het vanzelfsprekend was. Hiervoor ontzettend bedankt!

Marianne Schoorl bedankt voor de tomeloze inzet bij het verzamelen, analyseren en opslaan van alle bloedsamples voor de REDUCE studie. Het meedenken over welke bepalingen zinvol zijn en het uitzoeken wat daarvoor nodig is, heeft mijn werk aanzienlijk makkelijker gemaakt. Ook was het erg leerzaam om eens mee te draaien "achter de schermen" om te zien wat er allemaal bij komt kijken.

Wouter Rozemeijer en Wil van der Reijden bedankt voor de begeleiding en medewerking vanuit de microbiologie. Ik kan me nog goed herinneren hoe ik als onervaren onderzoeker bij jullie aan kwam kloppen om het te hebben over de microbiologie.
Ook hier was het kijkje "achter de schermen" zeer leerzaam en is het ook voor de dagelijkse praktijk erg nuttig geweest. In het bijzonder dank aan Wil voor de hulp bij het opzetten en uitvoeren van het qPCR onderzoek, vanaf het allereerste moment was je enthousiast en bereid om mee te werken, ook al moesten we het nog over de logistiek hebben. Dit is iets wat ik nooit zal vergeten en wat ik als uniek heb ervaren in mijn tijd als onderzoeker.

René Lutter, hartelijk dank voor het meedenken en meewerken aan het cytokine artikel. Als gewone dokter opeens weer terug de wereld in van de immunologie is niet makkelijk, maar uw begeleiding en uitleg hebben ervoor gezorgd dat er een mooi artikel tot stand is gekomen.

Beste Ted, Michael en Rosalie. Het was leuk en erg leerzaam om jullie te mogen begeleiden tijdens jullie wetenschappelijke stage. Ik ben er trots op dat jullie allemaal een artikel hebben weten te publiceren als eerste auteur. Twee van jullie hebben uiteindelijk besloten om zelf ook een promotietraject in te slaan, ik wens jullie veel succes!

Beste Henk-Jan, Nick, Lotte en Nienke, wat was het altijd gezellig op 117! De gezelligheid had niet altijd een positieve uitwerking op de snelheid van het onderzoek, maar heeft wel veel bijgedragen in het werkplezier. Henk-Jan, jij in het bijzonder bedankt voor mijn eerste stapjes in onderzoeksland toen ik mijn wetenschappelijke stage bij je deed. Je hebt me aan de hand meegenomen om me wegwijs te maken. De vele huisbezoeken bij de COPD patiënten die we samen gedaan hebben en hoe je altijd de mens achter de patiënt kon zien, staan voor altijd in mijn geheugen gegrift.

Bibi, Linda en Kelly jullie bedankt voor de ondersteuning bij de METC/Bureau wetenschap en alle gezelligheid!

Alle Alkmaarse, Zwolse en Amsterdamse longartsen wil ik graag bedanken voor de praktische hulp en medewerking. In het bijzonder wil ik nog Casper de Graaf bedanken, jij bent vanaf mijn allereerste begin in Alkmaar als onervaren ANIOS enorm betrokken geweest bij mijn ontwikkeling als dokter en als mens. Het siert je dat ik je ook na je pensioen altijd nog heb mogen benaderen voor advies en steun.

Ook dank aan alle longartsen uit het MCL, jullie hebben mij in de laatste jaren van het onderzoek en in de opleiding tot longarts veel steun geboden maar ook veel ruimte gegeven voor het onderzoek. Velen van jullie weten hoe het is om te promoveren naast een drukke klinische baan en alle extra tijd die ik van jullie gekregen heb is van onschatbare waarde geweest in het afronden van het proefschrift. In het bijzonder ook dank aan Wouter van Geffen voor jouw rol als opleider, het praktisch meedenken qua tijd en ruimte maken voor onderzoek maar ook de steun bij het volbrengen van de laatste loodjes.

Margreet en Erik, ontzettend bedankt! Niet alleen voor de hulp bij het maken en vormgeven van het boekje, maar ook de morele steun in het hele proces en het eindeloze geduld.

Beste Anneloes, je bent niet alleen tijdens de opleiding maar ook daarna altijd een steun en toeverlaat geweest. Ik hoop nog veel van je te mogen leren in de toekomst en omgekeerd jou te mogen steunen tijdens jouw promotietraject.

Lieve mam en pap, jullie hebben mij altijd met raad en daad bijgestaan en alle mogelijkheden geboden om zo ver te kunnen komen in het leven. Helaas kan pap dit niet meer meemaken, maar dit proefschrift is er getuige van dat hij een blijvend positieve invloed heeft gehad.

Tot slot, lieve Ellen, dank voor al je liefde, steun en geduld de afgelopen jaren. Dat je naast me staat bij de verdediging van dit proefschrift zegt meer dan genoeg.