Immunomodulation in community-acquired pneumonia

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Immunomodulation in community-acquired pneumonia

Modulatie van de immuunrespons bij een buiten het ziekenhuis verworven longontsteking
(met een samenvatting in het Nederlands)

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General introduction, aims and outline of the thesis

General introduction

Pneumonia is defined as an infection of the alveolar or gas-exchanging portions of the respiratory tract, with clinical symptoms (e.g. cough, fever, sputum production and pleuritic chest pain) accompanied by the presence of an infiltrate on chest radiography.[1] Worldwide, pneumonia is responsible for a considerable burden of morbidity and mortality. In the United States, along with influenza, pneumonia is the 8th leading cause of death in 2011 and is the leading cause of death from infectious diseases.[2] In the Netherlands, in 2003, the estimated incidence of pneumonia was 8.3 per 1000 men and 8.4 per 1000 women, with a higher incidence in children and elderly. In 2010, 35,409 persons were hospitalised because of pneumonia and 5413 persons died (31.3 per 100,000 men and 33.8 per 100,000 women).[3]

Pneumonia can be subdivided in community-acquired pneumonia (CAP), hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP). In CAP, which is the focus of this thesis, the most frequently identified microorganism is *Streptococcus pneumoniae*. Other commonly isolated microbes are *Haemophilus influenzae* and viruses.[4-6] In addition, with the development of improved microbiological techniques in the past years, the importance of *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Chlamydophila* species has become clear: the incidence of these microorganisms in CAP, commonly referred to as 'atypical pathogens', varies between 20% and 28%.[7] In the Netherlands, between 2007 and 2010, *Coxiella burnetii* was frequently encountered as causative microorganism of CAP due to a large outbreak of Q-fever.[8]

Disease severity in CAP is highly variable, and ranges from a mild illness to a life-threatening and - especially in elderly - potentially fatal disease. Clinical assessment by the attending physician often results in apparent underestimation of disease severity.[9] Several clinical scoring systems have been developed to assist the clinician to identify CAP patients with a poor prognosis at an early stage. Both the simple CURB-65 score and the more complex Pneumonia Severity Index (PSI) score are widely used in Europe and the USA to predict 30-day mortality.[10, 11] These scores are helpful management tools with regard to the need for hospitalisation and nursing level, the extent of microbiological diagnostics and the choice of empirical antibiotic treatment. A disadvantage of the PSI score is that it is highly influenced by age: young patients with severe

illness have a relatively low score. Furthermore, the prognostic performance of both the PSI score and CURB-65 score is not 100%. Therefore, additional studies are needed to investigate whether prediction of 30-day mortality can be improved by including new biomarkers.

Antimicrobials are the mainstay of CAP treatment and should be administered as soon as possible. As the causative microorganism is usually not known at presentation and cannot be reliably predicted from clinical features, initial antimicrobial treatment is empirical. Selection of empirical antibiotic therapy is based on knowledge of microbial patterns and local susceptibility profiles, as well as on disease severity upon presentation. Once the aetiology of CAP has been identified, antimicrobial treatment should be directed towards that pathogen.[1, 10, 12] In up to 50% of the cases, even when extensive microbial diagnostics are performed, no causative agent can be identified.[13, 14]

Despite the fact that potent antibiotics are available, CAP has remained a significant cause of morbidity and mortality over the years. Some patients with CAP still die from this disease, even with microbiological confirmation of adequate antibiotic therapy. This has led to increasing interest in non-antibiotic treatment options as adjuvant therapy in CAP. In this thesis, we focus on the immune response as target to improve outcome.

The immune response in community-acquired pneumonia

In everyday life, the respiratory tract is continuously exposed to microorganisms that are inhaled or aspired. Both the pathogenicity of the microbe and the integrity of host defence mechanisms determine whether infection will occur. Pulmonary defence mechanisms include mechanical barriers, such as ciliated epithelium and mucus, and the innate and acquired immune system (Figure 1).[15] The innate immune system provides immediate host defence, through cellular and humoral responses, is non-specific and (probably) lacks immunologic memory. The acquired immune response is characterised by antigen-specific reactions through B-lymphocytes and T-lymphocytes. This response takes time to develop, but has memory and therefore improves on repeated exposure to a given microbe. The innate and acquired immune system usually cooperate to eradicate pathogens.[16, 17] In this thesis, we focus on the innate immune response.

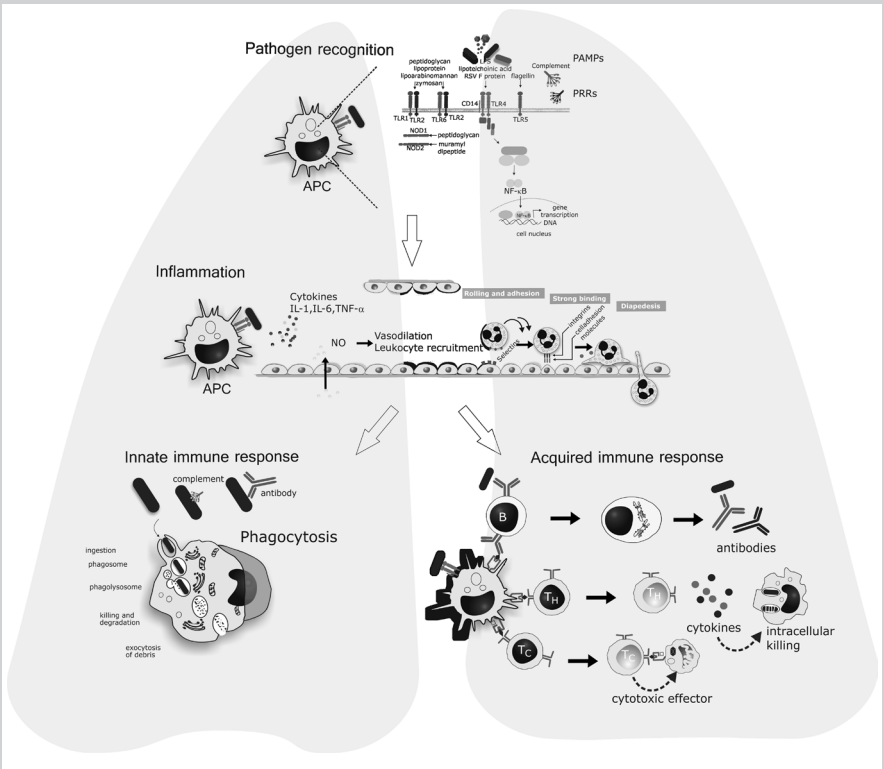

Figure 1. Overview of the immune response in community-acquired pneumonia. Abbreviations: APC, antigen presenting cell; PAMP, pathogen-associated molecular pattern; PRR, pathogen recognition receptor; IL, interleukin; NO, nitric oxide; TNF-α, tumour necrosis factor-α. Adapted with permission from S.C. Meijvis *et al.*[18]

The nature of the innate immune response depends on the type of microorganism that is encountered, the number of microorganisms, as well as the site of entrance. Extracellular microorganisms, mostly bacteria, that have managed to break through the mechanical barriers of the respiratory tract can be recognized by phagocytes (macrophages, monocytes and granulocytes). Pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), recognise 'pathogen-associated molecular patterns' (PAMPs) that are present on the cell surface of microbes. This interaction requires exposure to the cell surface of microbes and is therefore largely confined to extracellular microorganisms.[16] Most of the times, resident alveolar macrophages are sufficient to eradicate pathogens, at least to control the infection. However, when the microbial load is too large, too virulent, or if macrophage function

is impaired, additional phagocytes (predominantly neutrophils in bacterial CAP) are recruited from the pulmonary circulation to the site of infection.[15] The innate immune response against (facultative) intracellular microorganisms, such as *Mycoplasma pneumoniae* and viruses, mainly involves macrophages, natural killer (NK) cells and interferon-γ (IFN-γ).

Soluble mediators of innate defence include complement, acute-phase proteins and cytokines.[17] Complement activation, based on an enzymatic amplification cascade, results in the generation of several immunologically active proteins and protein fragments. Complement 'complements' the capability of phagocytes and antibodies to eliminate pathogens from the body, but can also cause lysis of pathogens by itself. Complement fragments are also potent chemoattractors for phagocytes. Acute-phase proteins are molecules that enhance resistance to infection and promote the repair of tissue injury. Examples include C-reactive protein (CRP), certain complement components and coagulation proteins.[17, 19] Cytokines are defined as the chemical messengers of the immune system that orchestrate the nature, intensity and duration of the immune response.[20] In general, they can initiate and amplify inflammatory and other immune responses by recruiting and activating cells, regulating the activation and differentiation of T- or B-lymphocytes in cell mediated immunity, and initiating and regulating local repair processes that are critical for the resolution of infection.[21] Cytokines can be produced by many cell types, for instance by activated alveolar macrophages. During infection, the synthesis of cytokines is stimulated by microbes or microbial products. Globally, we can categorise cytokines as either pro- or anti-inflammatory. Important pro-inflammatory cytokines in CAP are tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6.[22-25] Anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1ra) and IL-10, are induced to regulate the inflammatory response by inhibition of macrophage and neutrophil recruitment and activation.[18, 22, 25] Cytokines with chemo-attractive activity, such as IL-8 and monocyte chemoattractant protein-1 (MCP-1), are termed chemokines. They mobilise and activate neutrophils, and stimulate their degranulation.[16]
The local production of pro-inflammatory cytokines in the lung(s) contributes importantly to host defence during CAP. However, when excessive, it can and will lead to local tissue injury, which may contribute to the pathogenesis of acute respiratory distress syndrome (ARDS).[26] Moreover, systemic extension of the inflammatory response may lead to sepsis, multi-organ failure and even

death.[27] A careful balance between pro-inflammatory and regulatory anti-inflammatory responses, preventing an excessive inflammatory response, thus appears to be pivotal for a successful host response to infection.

Cortisol, the predominant glucocorticoid secreted by the adrenal cortex, is an important endogenous regulator of inflammation. Severe infection strongly activates the hypothalamic-pituitary-adrenal (HPA) axis, leading to an increased release of cortisol. Cortisol inhibits the activity of NF-κB, which plays a crucial role in inducing cytokine gene transcription. This results in decreased production of cytokines, chemokines and other inflammatory mediators. Next, cortisol reduces the number and function of various immune cells at the site of inflammation.[28]

Immunomodulation in community-acquired pneumonia

An unbalanced cytokine response, leading to an overwhelming pro-inflammatory state, is thought to be associated with adverse outcome in CAP. Prior studies in CAP have shown that clinical outcome is related to the extent of the inflammatory response.[22, 24] Therefore, it is postulated that attenuation of the immune response might be beneficial in treatment of CAP. An ideal intervention should restore the balance between pro- and anti-inflammatory immune responses, by dampening the pro-inflammatory response, and hence preventing or reducing systemic extension of inflammation. The ability to defend against invading pathogens and thus overcome the infection should remain intact. In this thesis, we investigate the immunomodulatory properties of corticosteroids, macrolides and vitamin D, and their ability to limit immunopathology in CAP.

Corticosteroids are potent inhibitors of the inflammatory response (Figure 2). Additionally, corticosteroids can restore an inadequate HPA axis response.[29, 30] Adjuvant corticosteroids have already been proven beneficial in a variety of infectious diseases, such as bacterial and tuberculous meningitis.[31-33] In CAP, the benefits of corticosteroids are less clear. Former intervention studies with corticosteroids in CAP have shown conflicting results.[34-40]

Macrolides are known to possess multiple immunomodulatory properties beyond their direct antibacterial activities, including alterations in leukocyte function, cytokine expression and mucus production (Figure 3).[18, 41] These immunomodulatory effects have already been proven beneficial in chronic

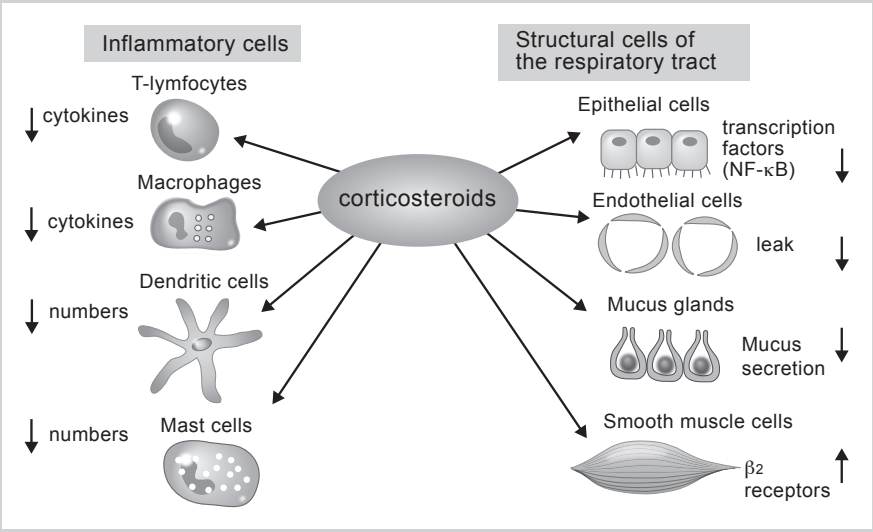

Figure 2. Overview of the effects of corticosteroids in community-acquired pneumonia. Adapted with permission from S.C. Meijvis *et al.*[18]

pulmonary inflammatory syndromes, such as diffuse panbronchiolitis and bronchiectasis.[42] Whether the immunomodulatory properties of macrolides also have favourable effects on pneumonia outcome is less clear, due to inconsistent study results up to now. Moreover, the current knowledge on the exact mechanisms of immunomodulation by macrolides during acute infection is limited and needs to be further explored.

Vitamin D is known to exert diverse immunomodulatory effects next to its classical role in calcium and bone homeostasis. Vitamin D plays a role in both innate and acquired immunity, by increasing the transcription of antimicrobial peptides and its effect on dendritic cells, monocytes, T-cells and B-cells, respectively (Figure 4).[43, 44] Vitamin D deficiency is associated with an increased risk of respiratory infections.[45-49] Several studies have investigated whether vitamin D supplementation can lower the risk of respiratory infections, however, with inconsistent results.[50-54] Thus, the role of vitamin D supplementation in prevention of CAP is still unclear.

Biomarkers in community-acquired pneumonia

Biomarkers are defined as biochemical features that can be used to diagnose or assess the progress of disease or the effects of treatment. Besides severity assessment, biomarkers can be helpful in many other areas of CAP, including:

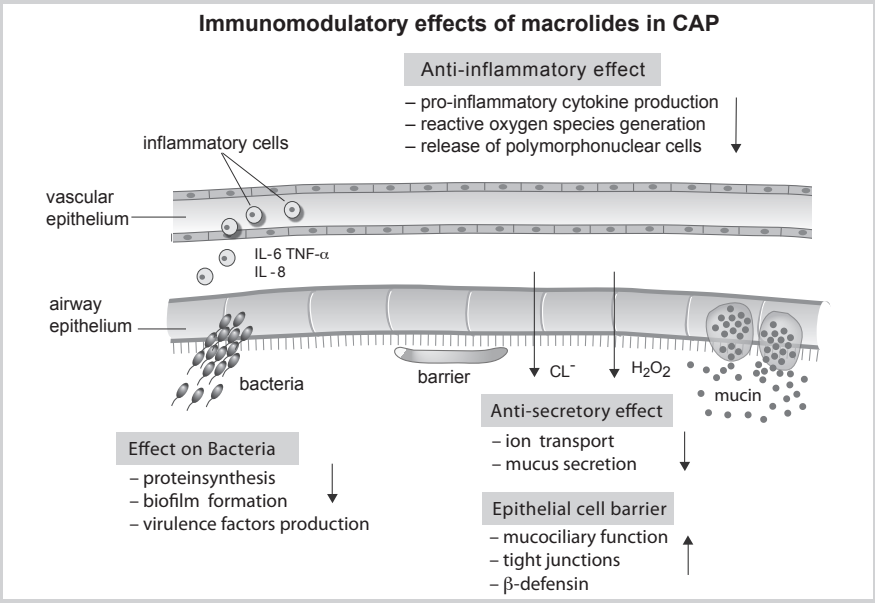

Figure 3. Overview of the effects of macrolides in community-acquired pneumonia. Adapted with permission from S.C. Meijvis *et al.*[18]

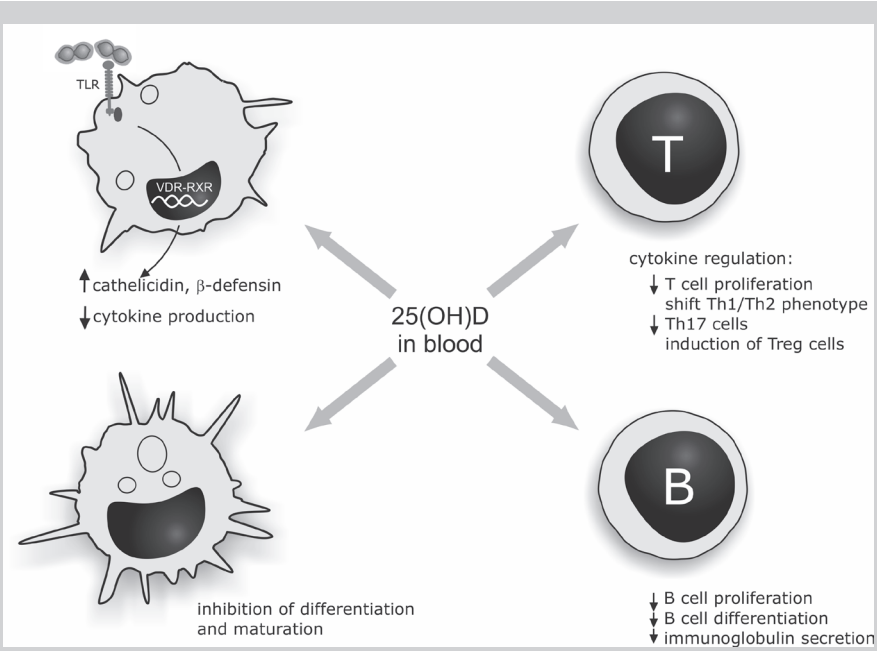

Figure 4. Overview of the immunomodulatory effects of vitamin D [25(OH)D] on macrophages / monocytes, dendritic cells, T-lymphocytes and B-lymphocytes. Based on data from M.F. Holick and C. Aranow.[43, 44]

confirmation of the diagnosis of CAP; identification of the aetiological origin of CAP, and hence, guidance for antibiotic treatment regimens; assessment of complication and mortality risk; and assessment of treatment effects during the course of disease.[55] The main biomarkers that have been studied in CAP are summarised in Table 1, classified by the (potential) field of application.[56, 57]

Table 1. Biomarkers in community-acquired pneumonia.

Indicators of diagnosis / aetiology	Leukocyte count
	C-reactive protein
	Procalcitonin
	Endotoxin
	Triggering receptor expressed on myeloid cells-1
Indicators of severity / prognosis	Leukocyte count
	C-reactive protein
	Procalcitonin
	Lactate
	Interleukin-6
	Interleukin-10
	Cortisol
	Copeptin (or Pro-vasopressin)
	(Pro-)adrenomedullin
	Pro-atrial natriuretic peptide
	(Pro-)B-type natriuretic peptide
	Pro-endothelin-1
	D-dimer
	Platelets
	Fibrinogen
	Activated partial thromboplastin time
	Protein C
Guide to therapy	Procalcitonin
	C-reactive protein

Next to these well-known application fields of biomarkers, biomarkers may be able to identify patients who might benefit from new adjuvant treatment options in CAP, such as corticosteroids. However, this has not been investigated yet. Additionally, only few data are available on the potential role of vitamin D as biomarker in CAP. Vitamin D is known to play a role in host defence against infection.[43] Previously, vitamin D deficiency has been shown to be associated with increased 30-day mortality in patients with CAP.[58] However, the potential of vitamin D as prognostic biomarker in CAP is currently unknown.

Aims and outline of the thesis

In this thesis, the immune response in CAP is further analysed. The effects of intervention with corticosteroids, macrolides and vitamin D on the immune response and clinical outcome are investigated. Furthermore, new biomarkers are identified in order to improve the prediction of prognosis and effectiveness of adjuvant therapy in CAP.

Part I focuses on the systemic inflammatory response triggered by the major causative microorganisms in CAP. In Chapter 2, a comprehensive panel of inflammatory markers was measured, in order to gain more insight into the host immune response to various pathogens.
Part II studies the role of adjuvant dexamethasone in the treatment of CAP. Chapter 3 reports the results of a randomised, placebo-controlled trial on the effect of dexamethasone on length of hospital stay in patients with CAP. Chapter 4 describes the influence of dexamethasone on the systemic cytokine response in patients with CAP, and whether this effect is dependent on causative microorganism. In Chapter 5, the association between cortisol levels and clinical outcome is investigated. Furthermore, the combination of cytokines and cortisol level as predictor of benefit from dexamethasone therapy is evaluated. Chapter 6 reports serial cortisol measurements during the clinical course of CAP. The association between serial cortisol levels and clinical outcome is studied, in order to map their potential as biomarker for prognosis. Furthermore, since secondary adrenal insufficiency is a potential risk of corticosteroid therapy, the time to recovery of the HPA axis after a short course of dexamethasone during CAP is studied.
Part III explores the potential role of macrolide antibiotics as immuno-modulatory agents in CAP. Chapter 7 reviews the available evidence from *in vitro* and *in vivo* studies on the immunomodulatory effects of macrolides during acute inflammation caused by pathogens commonly responsible for CAP. In literature, *in vitro* and *in vivo* studies evaluating the immunomodulatory effects of macrolides when combined with β-lactam antibiotics are lacking. A first attempt to fill this gap is made in Chapter 8. Here, a description is given of a series of experiments comparing the cytokine response during an *in vitro* model of acute infection with *Streptococcus pneumoniae* after treatment with different antibiotic regimens.
Part IV evaluates the role of vitamin D in CAP. In Chapter 9, first, the impact of vitamin D status on clinical outcome is studied. Second, the contribution of vitamin D status to the prognostic accuracy of other biomarkers and prognostic scores is investigated.

Vitamin D deficiency is known to be associated with an increased risk of developing respiratory tract infections. Whether vitamin D supplementation can lower the risk of developing pneumonia is uncertain. Therefore, in Chapter 10, three independent case control studies are conducted to explore the association between vitamin D supplementation and the risk of pneumonia. In Chapter 11, the summary and discussion of this thesis are presented, together with recommendations for future research.

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The immune response in community-acquired pneumonia

Pathogen-specific inflammatory response patterns in community-acquired pneumonia

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Submitted

Abstract

Background

Community-acquired pneumonia (CAP) is characterised by an inflammatory response. This study aimed to characterise inflammatory response patterns evoked by major causative microorganisms in CAP. We hypothesised that because different pathways of the host immune response need to be activated, extracellular microorganisms will require a different inflammatory profile than (facultative) intracellular microorganisms.

Methods

Subanalysis of two prospective studies including 469 adults admitted to the hospital with CAP. We selected three aetiological subgroups: patients with CAP caused by a mono-infection with *Streptococcus pneumoniae* (n=90), with a virus (n=34), or with an atypical pathogen (n=64). Serum concentrations of C-reactive protein (CRP), procalcitonin (PCT), leukocyte count and a panel of 11 cytokines/chemokines were measured on admission.

Results

Concentrations of PCT, leukocyte count, interleukin-6 (IL-6), IL-1 receptor-antagonist, IL-10, IL-17, interferon-γ and monocyte-chemotactic-protein-1 were significantly different among the three aetiological subgroups. Antibiotic use prior to admission did not change the inflammatory profiles. Within the group of atypical pathogens, also differences in the inflammatory response were found. In particular, *Legionella pneumophila* elicited the highest concentrations of CRP, PCT, leukocytes, IL-6, tumour necrosis factor-α, IL-10 and IL-8.

Conclusions

Major causative microorganisms in CAP trigger distinct inflammatory response profiles in the host. These profiles give more insight into the host immune response to various pathogens, and, in the future, can possibly assist in early identification of aetiology of CAP, or provide potential targets for adjuvant therapy.

Introduction

Community-acquired pneumonia (CAP) is a heterogeneous disease that can be caused by a broad range of microorganisms. CAP can present in patients of any age with a wide spectrum of clinical signs and symptoms, and can range in severity from a mild, sometimes self-limiting illness to life-threatening disease.[1, 2]
A common feature in all patients with CAP is the acute inflammatory response. Once pathogens have successfully passed the first line of defence (surface barriers, such as ciliated epithelium, mucus etcetera), the host initiates an immune reaction in an attempt to eliminate the invading microorganisms, beginning with the local release of a large number of inflammatory mediators, such as cytokines and chemokines.[3, 4] Because the local cytokine expression in the lungs is difficult to measure, the majority of research in patients with CAP has focused on the systemic extension of the immune response.
The most common causative pathogen in CAP is *Streptococcus pneumoniae*. Recognition of this microorganism by immune cells induces an array of cytokines. Cytokines that have been proven particularly important for the innate defence against pneumococci are tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and IL-18.[5] As causes of CAP, aside from *S. pneumoniae*, atypical bacteria (*Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydophila* species and *Coxiella burnetii*) and viruses are also frequent.[6] Given their capability of intracellular replication inside host cells, which contrasts with the extracellular replication of pneumococci, these pathogens are expected to trigger a different immune response in the host.
Indeed, a recent study by Menendez *et al*. of patients with CAP showed marked differences in the inflammatory response induced by the main causative microorganisms of CAP, giving each group of pathogens a specific profile.[7] They also reported that atypical bacteria triggered an inflammatory profile closer to that of viruses. However, this study investigated only a limited set of inflammatory markers (the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α, the regulatory cytokine IL-10, C-reactive protein (CRP) and procalcitonin (PCT)). In particular, it did not cover cytokines needed for cellular immunity against intracellular pathogens (such as interferon-γ (IFN-γ)).
Current knowledge from previous studies concerning pathogen specific inflammatory response patterns in CAP is restricted by the limited set of cytokines and chemokines that has been reported up to now.[7-13] The importance of other cytokines is unknown. Further elucidation of the pathogen-specific inflammatory response profiles in CAP is therefore needed. These profiles can

improve the current understanding of the host immune response to different microorganisms. Moreover, in the future, these profiles can possibly assist in early identification of the microbial aetiology of CAP, or may provide potential targets for adjuvant therapy.

The goal of the present study was thus to characterise the distinct inflammatory response patterns evoked by *S. pneumoniae*, atypical bacteria and viruses in CAP. To that end, in a well-defined cohort of CAP patients, we measured an elaborate panel of systemic inflammatory markers (CRP, PCT, leukocytes and 11 cytokines). We selected cytokines based on their target of action: those needed for innate immunity, for the strengthening of humoral immunity, as well as cytokines specifically needed for cellular immunity. We hypothesised that because different pathways of the host immune response need to be activated, extracellular microorganisms will require a different inflammatory profile than (facultative) intracellular microorganisms.

Methods

Study design and patients

For this cohort study, data were analysed from adults in The Netherlands who participated in two consecutive CAP studies. For both studies, the local Medical Ethics Committee approved the original studies and all patients gave written informed consent. In the first study, 201 patients with CAP were prospectively included between 2004-2006 to investigate the impact of polymorphisms of innate immunity genes and the dynamics of the systemic cytokine response in CAP.[14] In the second study, conducted between 2007-2010, 304 patients with CAP were randomly assigned to either a four-day course of dexamethasone or placebo to evaluate the effect of adjuvant dexamethasone therapy on length of hospital stay.[15] In both studies, inclusion criteria, pathogen identification methods and measurement of inflammatory markers were identical. The diagnosis of pneumonia was confirmed when a new pulmonary infiltrate was present on a chest radiograph, in combination with at least two of the following criteria: cough, sputum production, temperature greater than 38°C or lower than 35.5°C, auscultatory findings consistent with pneumonia, leukocytosis or leukopenia (>$10x10^9$ g/L or <$4x10^9$ g/L or >10% rods in leukocyte differentiation) and a CRP concentration of more than 15 mg/L. Patients who were immunocompromised or who received immunosuppressive therapy, including corticosteroids, were excluded from the current analysis. For study purposes, during their hospital stay, 50 percent of the patients from the second cohort received dexamethasone 5 mg once daily for 4 days. Before the first dose

of dexamethasone, blood samples were taken for inflammatory markers testing and microbiological diagnostic tests.

Pathogen identification

At least two sets of separate blood samples and sputum samples (if available) were cultured from each patient. Additionally, in-house developed polymerase chain reactions (PCR) were performed on the sputum to detect *L. pneumophila, M. pneumoniae, C. burnetii* and *Chlamydophila psittaci/pneumoniae*. Urinary antigen tests were performed to detect *L. pneumophila* serogroup 1 (Binax-Now; Binax, Portland, ME, USA) and *S. pneumoniae* (Binax-Now). Paired serological testing was performed for the presence of antibodies against *M. pneumoniae, C. burnetii, C. pneumoniae/psittaci* and respiratory viruses (adenovirus, influenzavirus A and B, parainfluenzavirus 1, 2 and 3 and respiratory syncytial virus) (Serodia, Bipharma, Fujirebio Inc, Tokyo, Japan), with a fourfold increase in antibody titer considered positive. Pharyngeal samples were taken for viral culture ((para-) influenza virus, adenovirus and respiratory syncytial virus).
Based on the results of the aforementioned microbiological tests, three specific subgroups of CAP patients were selected: 1) patients with CAP caused by a mono-infection with *Streptococcus pneumoniae,* 2) patients with CAP caused by a virus, and 3) patients with CAP caused by an atypical pathogen. Atypical pathogens included *L. pneumophila*, *M. pneumoniae*, *Chlamydophila* species and *C. burnetii*.

Measurement of inflammatory markers

Blood serum samples were collected on admission and stored at -80^{o}C until analysis. The conventional biomarkers CRP, leukocyte count and PCT were measured in all patients. Cytokines and chemokines were determined in the three aetiological subgroups of patients. Serum concentrations of CRP were determined with high sensitivity-CRP (Roche Diagnostics GmbH, Mannheim, Germany). Serum PCT concentrations were measured by electrochemiluminescence immunoassay (Elecsys Brahms PCT; Brahms GmbH, Hennigsdorf, Germany), with a lower detection limit of ≤0.02 ng/ml. Serum concentrations of interleukin-1 receptor antagonist (IL-1ra), IL-1β, IL-6, IL-8, IL-10, IL-12P40, IL-17, TNF-α, interferon-γ (INF-γ), macrophage inflammatory protein-1α (MIP-1α) and monocyte chemotactic protein-1 (MCP-1) were measured by Milliplex multi-analyte profiling (HCYTOMAG-60K-11, Millipore Corporation, Billerica, MA, USA) according to manufacturer's instructions. Data acquisition and analysis was performed on a Luminex 100 instrument (Luminex, Austin TX, USA).

Statistical analysis

All statistical analyses were performed using SPSS 18.0 (Chicago, IL, USA). A two-tailed p-value of <0.05 was considered significant, except where indicated. Baseline characteristics were compared among the aetiological subgroups using the Chi-square test, the Fisher's Exact-test or the one-way analysis of variance (ANOVA), where appropriate. For the analyses, because of a non-normal distribution, cytokine and PCT concentrations were transformed into a natural log scale. In the four cytokines in which logarithmic transformation did not result in a normal distribution, dichotomisation was performed based on the medians. Comparisons of the concentrations of conventional biomarkers and cytokines/chemokines among the three aetiological subgroups of interest (mono-infection with *S. pneumoniae*, virus or an atypical pathogen) were carried out by one-way ANOVA for continuous variables, or by the Chi-square test for dichotomised variables. Because multiple outcome parameters were tested in this comparison analysis, which gives a higher risk of Type I error, α was set at 0.01. When a significant difference was found between groups, *post-hoc* pairwise comparisons were performed with the Bonferroni correction.

Regression analysis (logistic or linear, where appropriate) was performed to examine whether antibiotic treatment prior to admission could have confounded the relation between aetiological subgroups and the inflammatory response. Confounding was defined as modification of the regression coefficient by ≥10%. The variable 'antibiotic treatment prior to admission' was included in the multivariate regression model next to the variable 'aetiological groups' (both independent variables) to predict the concentrations of the inflammatory markers on day 0 (dependent variable).

Results

Study population

In total, 469 patients were included in this study. The mean age of the patients was 62.8 years (SD 18.3) and 58% were male. The mean Pneumonia Severity Index (PSI) score was 88 (SD 36) and 203 patients (43%) were classified as PSI class IV or V. During hospital stay, 36 patients (7.7%) were admitted to the ICU, of which 5 patients (1.1%) immediately on presentation, and 24 patients (5.1%) died.

An aetiological diagnosis could be established in 271 patients (58%). 188 patients (40%) were selected with a definitive diagnosis of CAP caused by a mono-infection with either *S. pneumoniae* (n=90, 19%), an atypical pathogen

(total n=64, 14%; *Chlamydophila* species, n=13, 2.8%; *L. pneumophila,* n=20, 4.3%; *C. burnetii*, n=25, 5.3%; *M. pneumoniae*, n=6, 1.3%) or a virus (n=34, 7.2%). The baseline characteristics for the whole study population, as well as the comparison of baseline characteristics among the three aetiological subgroups, are given in Table 1. Overall, atypical infections were more common in younger patients and *S. pneumoniae* pneumonia was more common in COPD patients.
In addition to Table 1, for the atypical pathogens separately, the percentage of patients presenting with systemic inflammatory response syndrome (SIRS) and with severe pneumonia, as indicated by PSI class IV or V, is as follows: *Chlamydophila* species, SIRS in 100% and PSI IV-V in 31% of the cases; *L. pneumophila,* SIRS in 100% and PSI IV-V in 55% of the cases; *C. burnetii,* SIRS in 78% and PSI IV-V in 16% of the cases; *M. pneumoniae,* SIRS in 50% and PSI IV-V in 0% of the cases.

Leukocyte, C-reactive protein and procalcitonin response among aetiological subgroups

The biomarker concentrations among the three aetiological subgroups are shown in Table 2. Compared to patients with CAP caused by an atypical pathogen or a virus, patients with pneumococcal pneumonia showed significantly higher leukocyte counts (p:0.000) and PCT concentrations (p:0.000). Those with atypical pathogens and viruses exhibited a comparable PCT response. Patients with CAP caused by an atypical pathogen had the lowest leukocyte and PCT concentrations, while patients with CAP caused by a virus had the lowest concentrations of CRP. Pairwise comparisons for biomarker concentrations among the three subgroups are shown in Figure 1A. Subsequent analysis of the atypical pathogens separately revealed differences in CRP, leukocyte count and PCT dependent on the atypical pathogen (Table 3). *L. pneumophila* elicited the highest leukocyte counts, CRP and PCT concentrations, while in *M. pneumoniae* pneumonia PCT concentrations were just barely above background concentrations.

Cytokine profiles among aetiological subgroups

The three aetiological subgroups of CAP showed different, characteristic cytokine and chemokine profiles (Figure 2). Comparison of the cytokine and chemokine concentrations among the subgroups is shown in Table 2. Patients with pneumococcal pneumonia displayed significantly higher IL-6, IL-1ra, and MCP-1 concentrations than did patients with CAP caused by a virus or an atypical pathogen. Patients with CAP caused by an atypical pathogen had significantly

Table 1. Baseline characteristics of the three aetiological subgroups of patients with community-acquired pneumonia.

Characteristics	All patients (n=469)	*S. pneumoniae* (n=90)	Atypical pathogen[a] (n=64)	Virus (n=34)	p-value[b]
Sex, no. of males (%)	270 (57.6)	44 (48.9)	41 (64.1)	25 (73.5)	0.025[c]
Age, in years (SD)	62.8 (18.3)	60.7 (19.5)	54.0 (15.6)	69.2 (18.1)	0.000[c]
Comorbidities					
Neoplastic disease (%)	39 (8.3)	9 (10.0)	2 (3.1)	2 (5.9)	0.25
Congestive heart failure (%)	59 (12.6)	7 (7.8)	7 (10.9)	5 (14.7)	0.50
Renal disease (%)	37 (7.9)	5 (5.6)	3 (4.7)	6 (17.6)	0.07
Diabetes mellitus (%)	70 (14.9)	15 (16.7)	4 (6.3)	6 (17.6)	0.10
COPD (%)	68 (14.5)	20 (22.2)	2 (3.1)	5 (14.7)	0.002[c]
PSI score (SD)	88.1 (36.4)	87.4 (37.0)	76.9 (35.1)	94.9 (35.3)	0.048[c]
PSI risk class					0.10
Class I-III (%)	266 (56.7)	51 (56.7)	45 (70.3)	17 (50.0)	
Class IV-V (%)	203 (43.3)	39 (43.3)	19 (29.7)	17 (50.0)	
SIRS present (>1 criteria) (%)	380 (81.0)	78 (89.7)	52 (86.7)	26 (83.9)	0.62
Antibiotics prior to admission (%)	122 (26.0)	16 (17.8)	21 (33.3)	8 (23.5)	0.09
Outcome					
ICU admittance (%)	36 (7.7)	8 (8.9)	4 (6.3)	1 (2.9)	0.55
In-hospital mortality (%)	24 (5.1)	3 (3.3)	1 (1.6)	2 (5.9)	0.40

Data are presented as number (%) or mean (SD, standard deviation).

[a] Atypical pathogens: *Legionella pneumophila, Mycoplasma pneumoniae, Chlamydophila* species and *Coxiella burnetii*.

[b] Comparison of the three aetiological subgroups.

[c] Characteristics showing a significant association with a p-value <0.05.

Abbreviations: COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; PSI, pneumonia severity index; SIRS, systemic inflammatory response syndrome.

Table 2. Biomarker and cytokine concentrations among three aetiological subgroups of patients with community-acquired pneumonia.

Inflammatory marker	S. pneumoniae (n=90)	Atypical pathogen[a] (n=64)	Virus (n=34)	p-value[b]
Leukocytes (x109/L) (SD)	17.1 (7.5)	11.0 (4.0)	13.1 (5.9)	0.000[c]
CRP (mg/L) (SD)	289.5 (164.7)	256.9 (110.9)	208.3 (119.9)	0.02
PCT (ng/ml) (IQR)	2.3 (0.4-8.1)	0.3 (0.1-1.1)	0.4 (0.2-1.4)	0.000[c]
IL-1β <1.58 pg/ml (no. %) >1.58 pg/ml (no. %)	41 (46.6) 47 (53.4)	28 (45.9) 33 (54.1)	9 (26.5) 25 (73.5)	0.11[d]
IL-6 (pg/ml) (IQR)	155.7 (36.7-746.4)	31.9 (14.4-93.5)	57.0 (15.7-121.9)	0.000[c]
IL-12P40 <1.31 pg/ml (no. %) >1.31 pg/ml (no. %)	34 (38.6) 54 (61.4)	12 (19.7) 49 (80.3)	13 (38.2) 21 (61.8)	0.04[e]
TNF-α (pg/ml) (IQR)	11.7 (6.1-26.3)	11.8 (6.4-18.9)	11.1 (8.5-14.2)	0.67
IL-8 (pg/ml) (IQR)	29.1 (13.1-82.5)	17.9 (12.4-36.8)	30.9 (19.3-50.7)	0.27
MCP-1 (pg/ml) (IQR)	621.7 (348.1-1704.1)	314.1 (218.6-474.6)	436.0 (333.6-709.4)	0.000[c]
MIP-1α <1.81 pg/ml (no. %) >1.81 pg/ml (no. %)	67 (76.1) 21 (23.9)	52 (85.2) 9 (14.8)	29 (85.3) 5 (14.7)	0.29[f]
IL-1ra (pg/ml) (IQR)	20.9 (3.2-152.1)	3.2 (2.2-22.2)	3.2 (1.8-37.3)	0.001[c]
IL-10 (pg/ml) (IQR)	18.3 (3.3-56.9)	1.8 (1.3-7.7)	9.6 (2.3-55.1)	0.000[c]
IL-17 <1.59 pg/ml (no. %) >1.59 pg/ml (no. %)	43 (48.9) 45 (51.1)	12 (19.7) 49 (80.3)	15 (44.1) 19 (55.9)	0.001[cg]
IFN-γ (pg/ml) (IQR)	2.1 (1.5-9.2)	31.8 (6.8-62.9)	2.4 (1.5-15.3)	0.000[c]

Data are presented as mean (SD, standard deviation), median (IQR, interquartile range), or number (%).
[a] Atypical pathogens: *Legionella pneumophila, Mycoplasma pneumoniae, Chlamydophila* species and *Coxiella burnetii*.
[b] Comparison of the three aetiological subgroups.
[c] Characteristics showing a significant association with a p-value <0.01.
[d] IL-1β <1.58 (median) as cut-off to compare the three aetiological subgroups
[e] IL-12P40 <1.31 (median) as cut-off to compare the three aetiological subgroups
[f] MIP-1α <1.81 (median) as cut-off to compare the three aetiological subgroups
[g] IL-17 <1.59 (median) as cut-off to compare the three aetiological subgroups
Abbreviations: CRP, C-reactive protein; PCT, procalcitonin; IL, interleukin; MCP, monocyte-chemotactic protein; MIP, macrophage inflammatory protein; TNF, tumour-necrosis-factor; IFN, interferon.

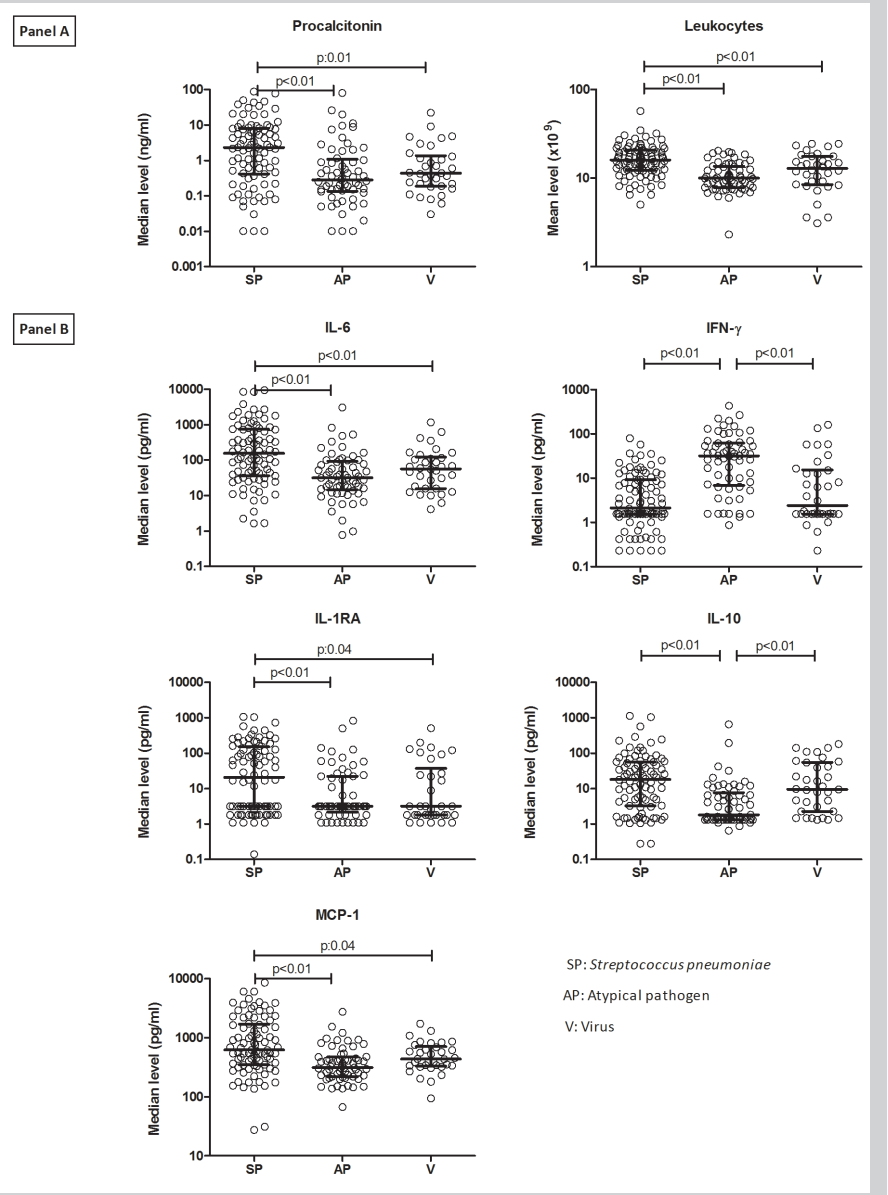

Figure 1. Pairwise comparisons (with Bonferroni correction) of the median concentrations (with interquartile ranges) or mean concentrations (with standard deviation) of the conventional biomarkers (Panel A) and cytokines/chemokines (Panel B) that showed significant differences among the three aetiological subgroups of patients with community-acquired pneumonia. Significant results (p<0.05) are indicated. Abbreviations: IL, interleukin; IL-1RA ; MCP, monocyte-chemotactic protein.

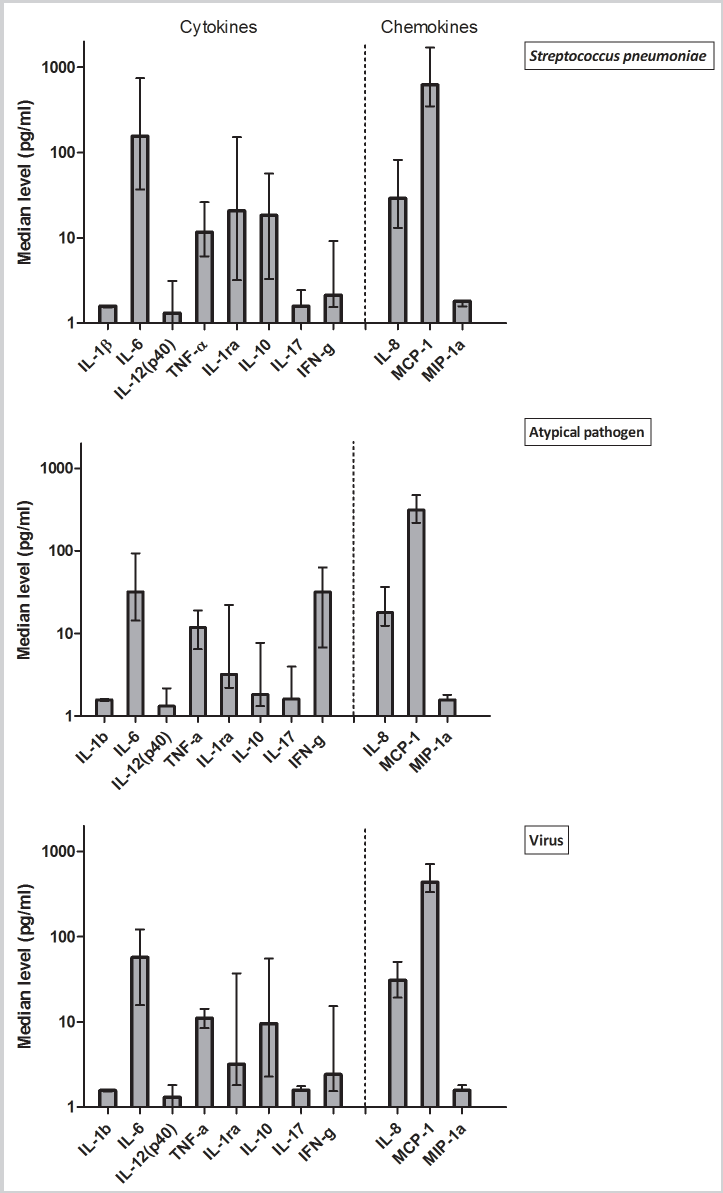

Figure 2. Overview of cytokine and chemokine profiles upon hospital admission with community-acquired pneumonia of different aetiologies. Median concentrations with interquartile ranges are shown. In healthy individuals, serum cytokine concentrations are generally below 10 pg/ml. For chemokines, higher concentrations have been reported: IL-8 (23.9-77.8 pg/ml), MCP-1 (168.0-213.5 pg/ml) (MIP-1α concentrations in healthy individuals have not been reported to date).[22,23] Abbreviations: IL, interleukin; MCP, monocyte-chemotactic protein; MIP, macrophage inflammatory protein; TNF, tumour-necrosis-factor; IFN, interferon.

higher IL-17 and IFN-γ concentrations than did patients with CAP caused by other microorganisms. Moreover, patients with an atypical pathogen had the lowest concentrations of IL-10. Pairwise comparisons of the concentrations of IL-6, MCP-1, IL-1ra and IFN-γ among the three subgroups are shown in Figure 1B. Analysis of the cytokine and chemokine concentrations evoked by the atypical pathogens separately revealed discrete differences (Table 3). For example, *L. pneumophila* showed the highest concentrations of IL-6, TNF-a, IL-10 and IL-8. Compared to other atypical pathogens, *Chlamydophila* species elicited a considerably higher concentration of IL-12P40. *M. pneumoniae* showed the lowest concentration of IFN-γ.

The influence of antibiotics prior to admission on the inflammatory response

In total, receipt of antibiotics prior to hospital admission was true for 45 of 188 patients (24%). We analysed whether this could have confounded the differences in inflammatory response among the aetiological groups. When we corrected for antibiotic use prior to admission in the comparison analyses, it did appear to be a confounder in the relationship between the aetiological subgroups and the concentration of IL-1ra (13% modification of the regression coefficient in the comparison 'atypical pathogen versus virus': -1.1678 (95% CI -2.0144 - -0.3212) versus -1.1530 (95% CI -2.0007 - -0.3054)). Nevertheless, despite this confounding influence, aetiological origin remained an independent risk factor for the concentration of IL-1ra (corrected p-value: 0.003). For the other inflammatory markers, correction for antibiotic use prior to admission did not change the results (data not shown). This confirmed that the inflammatory profile in CAP is determined by the causative microorganism and not significantly influenced by antibiotic treatment prior to admission.

Table 3. Biomarker and cytokine concentrations among the individual atypical pathogens. Data are presented as mean (standard deviation, SD) or median (interquartile range, IQR).

Inflammatory marker	*Chlamydophila* species (n=13)	*Legionella pneumophila* (n=20)	*Coxiella burnetii* (n=25)	*Mycoplasma pneumoniae* (n=6)
Leukocytes (x10^9/L) (SD)	10.5 (4.8)	13.6 (4.4)	9.6 (2.0)	9.2 (3.0)
CRP (mg/L) (SD)	279.9 (99.2)	307.5 (86.1)	240.0 (112.9)	108.7 (52.1)
PCT (ng/ml) (IQR)	0.4 (0.3-2.5)	0.9 (0.2-2.8)	0.2 (0.1-0.5)	0.04 (0.01-0.06)
IL-1β (pg/ml) (IQR)	1.6 (1.5-1.6)	1.6 (1.5-1.6)	1.5 (1.5-1.6)	1.6 (1.2-1.6)
IL-6 (pg/ml) (IQR)	28.7 (18.9-90.8)	101.8 (38.5-161.7)	26.7 (12.5-47.0)	9.1 (1.7-16.3)
IL-12P40 (pg/ml) (IQR)	6.2 (1.4-13.7)	1.3 (0.2-1.4)	1.3 (1.3-1.4)	1.3 (0.2-2.4)
TNF-α (pg/ml) (IQR)	11.9 (9.3-22.0)	16.7 (6.5-22.0)	10.8 (6.5-15.9)	6.4 (4.2-9.3)
IL-8 (pg/ml) (IQR)	24.4 (15.4-31.0)	35.7 (15.4-59.5)	14.3 (10.8-28.6)	10.9 (8.2-14.6)
MCP-1 (pg/ml) (IQR)	398.7 (300.4-425.2)	399.7 (219.7-894.1)	262.6 (167.3-475.5)	275.3 (164.7-390.1)
MIP-1α (pg/ml) (IQR)	1.5 (1.6-2.4)	1.5 (1.6-1.8)	1.6 (1.6-1.6)	1.7 (1.6-1.8)
IL-1ra (pg/ml) (IQR)	12.7 (1.5-33.9)	6.4 (1.8-111.2)	3.2 (3.2-3.2)	2.5 (1.6-7.5)
IL-10 (pg/ml) (IQR)	3.3 (1.5-11.9)	5.6 (1.6-13.2)	1.3 (1.3-3.9)	1.6 (1.4-5.9)
IL-17 (pg/ml) (IQR)	1.6 (1.6-4.8)	1.6 (1.6-3.3)	1.6 (1.6-5.4)	1.6 (1.5-6.3)
IFN-γ (pg/ml) (IQR)	43.8 (1.6-103.1)	44.1 (13.0-119.1)	32.1 (8.6-40.1)	6.0 (3.3-18.0)

Abbreviations: CRP, C-reactive protein; PCT, procalcitonin; IL, interleukin; MCP, monocyte-chemotactic protein; MIP, macrophage inflammatory protein; TNF, tumour-necrosis-factor; IFN, interferon.

Discussion

In this study, we identified specific profiles of inflammatory markers for the most common causative microorganisms in CAP. Significant differences in the concentration of PCT, leukocyte count, IL-6, MCP-1, IL-1ra, IL-10, IL-17 and IFN-γ were found among patients with CAP caused by *S. pneumoniae*, by an atypical pathogen or by a virus. Antibiotic use prior to admission did not change the inflammatory profiles. Another major finding was the difference in inflammatory profiles within the group of atypical pathogens.

Recently, Menendez *et al*. were the first to report specific profiles for the most common pathogens causing CAP.[7] Their evaluation was limited to a restricted panel of inflammatory markers. In the current study, the pathogen-specific inflammatory profiles in CAP have been confirmed and expanded.

Cytokine profile - The cytokine profile for pneumococcal pneumonia is characterised by high concentrations of the pro-inflammatory cytokines TNF-α and IL-6, the anti-inflammatory cytokines IL-1ra and IL-10, and the chemokines IL-8 and MCP-1. Compared to CAP caused by an atypical pathogen or a virus, pneumococcal pneumonia showed considerably higher concentrations of IL-6, IL-1ra and MCP-1. The finding of elevated IL-6 and IL-10 concentrations in pneumococcal pneumonia is in line with the recent study of Zobel *et al*.[13] The high concentrations of IL-10 and IL-1ra most probably reflect the requirement for regulation of the severe pro-inflammatory response. As expected, the cytokine profile found for pneumococcal pneumonia was mainly targeted at the killing of extracellular microorganisms by innate immune defence mechanisms and antibody formation (humoral immunity).[5] The cytokine profile of CAP caused by atypical pathogens contrasts with the profile of pneumococcal and viral CAP by exhibiting a high IFN-γ concentration and, to a lesser extent, a higher concentration of IL-17. High IFN-γ concentrations are compatible with induction of a T-helper type 1 (Th1) cell-mediated inflammatory response to kill the atypical pathogens, which is in line with the (facultative) intracellular nature of these microorganisms.[16] The involvement of IL-17 in atypical pneumonia has previously been demonstrated by Kimizuka *et al*., who showed that IL-17 plays a critical role in *L. pneumophila* pneumonia, probably through induction of pro-inflammatory cytokines and accumulation of neutrophils at the site of infection.[17]

While Menendez *et al*. reported that atypical bacteria exhibited an inflammatory profile closer to that of viruses, we found clear differences in the cytokine profile between atypical bacteria and viruses.[7] In particular, concentrations of IFN-γ, IL-

10 and IL-17 in CAP caused by an atypical pathogen appeared to be distinct from those in viral CAP.

Profile of conventional biomarkers - Next to these pathogen-specific cytokine patterns, we also found specific profiles of the more commonly used biomarkers among the three aetiological subgroups. *S. pneumoniae* provoked highest leukocyte counts, PCT and CRP concentrations. Viruses showed lowest CRP concentrations. Atypical pathogens and viruses showed lowest PCT concentrations. These findings are in accordance with previous studies that investigated inflammatory biomarkers in patients with CAP of different etiologies.[7, 11, 12]

An accumulation of data now indicates that serum PCT can be an accurate marker for differentiation between bacterial and viral pneumonia, and it has also been proposed that PCT concentrations could guide the decision to initiate antibiotic therapy.[18-20] It thus is suggested that antibiotic treatment can be withheld in case of low PCT concentrations upon admission with CAP. Our data, which are in accordance with others, show that PCT concentrations can be low in CAP caused by an atypical (but bacterial!) pathogen. The only exception was CAP caused by *L. pneumophila,* in which a higher PCT concentration was found. From these data we can conclude that PCT is able to discriminate between CAP caused by typical bacteria and CAP caused by a virus or an atypical pathogen. However, PCT is unable to distinguish CAP caused by an atypical pathogen from viral CAP. This distinction is particularly important in clinical practice because of the therapeutic consequences. In contrast with patients with viral CAP, those with CAP caused by an atypical pathogen do need appropriate antibiotic treatment. This is underscored by our cohort of hospitalised patients with CAP, which includes a considerable number of patients with CAP caused by an atypical pathogen. Overall, in these patients, a systemic inflammatory response syndrome (SIRS) was present in 87%, and 30% of the patients were classified as PSI class IV-V (Table 1), although considerable differences between the individual atypical pathogens exist (see Results section). If the decision to initiate antibiotic treatment should be based on the PCT value on admission, up to 14% of CAP patients would not receive the most appropriate clinical care. If the decision to initiate antibiotic treatment would have to be based on a biomarker profile, our study suggests that, in addition to PCT, IFN-γ, IL-17 or IL-10 (in that order) could be added. Clearly, further studies are needed to investigate the diagnostic value of these markers and the eventual clinical application.

While previous studies merely investigated atypical pathogens as a group, we analysed the atypical pathogens both as a group and separately. We found considerable differences in inflammatory response within the group of atypical pathogens. In particular, CAP caused by *L. pneumophila* showed remarkably higher concentrations of PCT, leukocyte count, CRP, IL-6, TNF-α, IL-10 and IL-8 compared to the other three atypical pathogens. A possible explanation can be the facultative intracellular nature of *L. pneumophila*, which allows both intracellular and extracellular growth of the microorganism. As a result, *L. pneumophila* might trigger another inflammatory response than obligate intracellular microorganisms. Furthermore, the concentration of IL-12P40 was higher in *Chlamydia* spp. than in the other three atypical pathogens. Although the number of patients in these subgroups was relatively low, the data emphasize the heterogeneity of the inflammatory response in CAP and suggest that in future studies these microorganisms should be investigated individually rather than lumping them together.

In clinical practice, there are several possible applications of the pathogen-specific inflammatory patterns. Pathogen-specific inflammatory patterns can possibly assist in the early identification of the microbial aetiology of CAP, and hence, can improve guidance of empirical antibiotic therapy, while awaiting the results of microbial diagnostics. Next, pathogen-specific inflammatory patterns may provide potential targets for adjuvant therapy. Since CAP can be caused by a broad range of microorganisms, different inflammatory patterns can be encountered and potentially be matched with immunomodulatory therapies that influence the immune response in a specific way.[4] Pathogen-specific inflammatory patterns can possibly provide insight in which patients with CAP will benefit from certain adjuvant therapies, which may lead to improved application of adjuvant immunomodulatory therapies.

Although our study provides insight into the pathogen-specific inflammatory profiles and has tested a large set of systemic cytokines in a well defined cohort of CAP patients, it has not included all identifiable cytokines. Instead, preliminary experiments screened a panel of 27 cytokines and chemokines, from which we selected the 11 cytokines upregulated during CAP. Further, only systemic cytokine measurements were performed in this study. While the systemic cytokine response in CAP might differ from the local cytokine response in the lung(s), in daily practice such systemic measurements are usually the only possibility. It is also clear that because most cytokines have a short half-life time, and patients present to the hospital at different stages of disease, a true day zero is lacking.[21] This might have influenced the inflammatory profiles

that we have found. Finally, despite the extensive microbiological workup, an aetiological diagnosis was established in only 58% of our patients. This resulted in a lack of statistical power for the atypical pathogens separately. Larger studies are needed to map the inflammatory patterns for the causative microorganisms in CAP individually.

In conclusion, this study further elucidated the pathogen-specific inflammatory patterns in CAP. Specific inflammatory profiles were found for *S. pneumoniae,* atypical pathogens and viruses. These profiles give more insight into the host immune response to various microorganisms commonly causing CAP. The applicability of these profiles in clinical practice remains to be defined in future studies, with larger studies needed to allow individual analysis of the pathogen-specific inflammatory patterns for all microorganisms.

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II

Immunomodulation by dexamethasone

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

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Abstract

Background

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

Methods

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

Results

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

Conclusions

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

Introduction

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

In CAP, locally produced pulmonary cytokines are needed to control and eliminate the primary infection. However, organ dysfunction can result from a systemic inflammatory response.[3] Therefore, a balanced cytokine response needs to be sufficient to control the local infection but not be excessive to prevent systemic effects. An ideal intervention would reduce the systemic complications of the inflammatory response without affecting the resolution of local inflammation.

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

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

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

Methods

Study design and patients

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

Randomisation and masking

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

Procedures

Patients in the dexamethasone group were given a bolus of 5 mg (1 mL) of dexamethasone (dexamethasonedisodiumphosphate 5 mg, Centrafarm BV, Etten-Leur, Netherlands) intravenously and patients in the placebo group were given 1 mL of sterile water for injection (Centrafarm BV) intravenously at

the emergency unit, within a maximum of 12 h from admission. All patients received antibiotics before study treatment was given. For the subsequent 3 days, patients received either intravenous dexamethasone 5 mg (1 mL) or sterile water (1 mL) once a day. Selection, duration and administration of the antibiotic treatment were decided by the medical team in charge and were done according to national guidelines.[12] The decision to transfer a patient to the intensive care unit or hospital discharge were established by their medical team. A general rule for hospital discharge in both hospitals was that patients were clinically stable (improvement of shortness of breath, absence of hyperthermia or hypothermia, consistent decrease of C-reactive protein concentrations, and adequate oral intake and gastrointestinal absorption) and be in a condition to leave the hospital.

The primary endpoint was length of hospital stay in days until hospital discharge or death. If a patient was admitted between 2400 h and 1200 h, the day of admission was counted as 1 day; if the patient was admitted after 1200 h, the day of admission was counted as 0.5 days. Secondary endpoints included mortality, admissions to intensive care units, development of empyema, superinfection, readmission, time-courses of C-reactive protein, interleukin-6, and interleukin-10 concentrations, pulmonary function at day 30 and general health-related quality of life as measured by the RAND-36 generic health survey (Appendix A).[13] Pleural effusion was defined as pleural fluid layer thickness on chest radiograph of more than 1 cm that needed additional assessment (i.e., pleural puncture) and empyema was defined as pleural effusion containing bacteria. A superinfection was defined as a new infection with or without the need for antibiotic treatment. Readmission was defined as admission to hospital within 30 days from discharge. At a control visit 30 days after the day of admission, lung function was assessed by body plethysmography and carbon monoxide diffusion and helium dilution. Measurements were done in the pulmonary function laboratory of the hospital in which the patient was admitted. Other secondary objectives that were prespecified in the study protocol are beyond the scope of this report and will be reported elsewhere.

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

on the day of presentation and days 1, 2, and 4, and at the control visit. At admission, we measured total serum cortisol concentrations in blood drawn before administration of the study medication with an ELISA kit (Calbiotech, Spring Valley, CA, USA). Appendix B describes the method used for pathogen identification. Treating doctors assessed comorbidities (neoplastic disease, liver disease, congestive heart failure, renal disease, diabetes mellitus and chronic obstructive pulmonary disease [COPD]). We calculated a pneumonia severity index score for all patients.[14]

Statistical analysis

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

treatment. Differences between the two treatment groups were analysed with the χ^2 test. All statistical analyses were done with SPSS version 15.0. A two-tailed p-value of less than 0.05 was regarded as significant, apart from multiple comparisons of the quality of life items, in which we used a conservative value of p<0.01. Interim analyses were preplanned and done after the inclusion of 100 and 200 patients to assess the frequency of serious side effects related to either dexamethasone or placebo. An external, independent data and safety monitoring board reviewed the results of these interim analyses.

Results

From November, 2007, to September, 2010, we enrolled 304 patients (Figure 1, Table 1). 133 (44%) patients had comorbidities, with more patients having renal disease in the dexamethasone group than in the control group (Table 1). 79 (52%) of 151 patients in the dexamethasone group were in pneumonia severity index risk classes 4 and 5 compared with 64 (42%) of 153 in the placebo

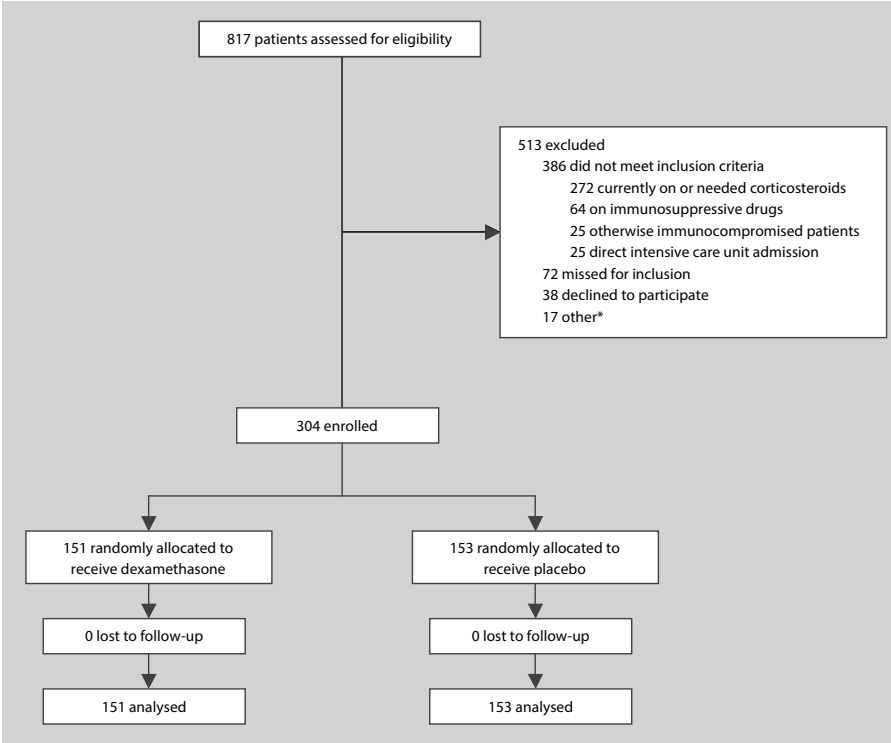

Figure 1. Study profile.
* E.g., pregnant or breastfeeding.

Table 1. Baseline characteristics of enrolled patients

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

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

group (Table 1). Baseline characteristics of patients did not differ between the two hospitals (data not shown).

For the primary outcome, the median length of hospital stay in the dexamethasone group was 6.5 days (IQR 5.0–9.0) compared with 7.5 days (5.3–

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

Table 2. Outcomes for all enrolled patients

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

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

All patients were treated with intravenous antibiotics within 4 h of admission to hospital according to national guidelines.[12] Antibiotic treatment was much the same in both groups (Appendix C). 18 (12%) of 151 patients in the dexamethasone group and 16 (10%) of 153 patients in the placebo group were treated with a macrolide alone or as part of combination therapy. Antibiotic treatment was modified on the basis of the outcome of the microbiological investigation. The mean time of switching to oral administration of antibiotics was 5.0 days (SD 4.2) in the dexamethasone group and 5.1 days (3.5) in the placebo group. We established the microbial cause of CAP in 168 (55%) of 304 patients (Appendix D). *Streptococcus pneumoniae, Coxiella burnetii, Chlamydophila* spp, and *Legionella* spp were the most frequently identified microorganisms. Distribution of the pathogens did not differ between groups. We noted mixed infection in 21 (7%) patients. 132 (87%) of 151 patients in the dexamethasone group and 134 (88%) of 153 patients in the placebo group completed the 4-day course of study treatment. 13 patients did not complete the course because of admission to intensive care units, four died, and 21 had protocol violations (Appendix E). For secondary outcomes, hospital mortality

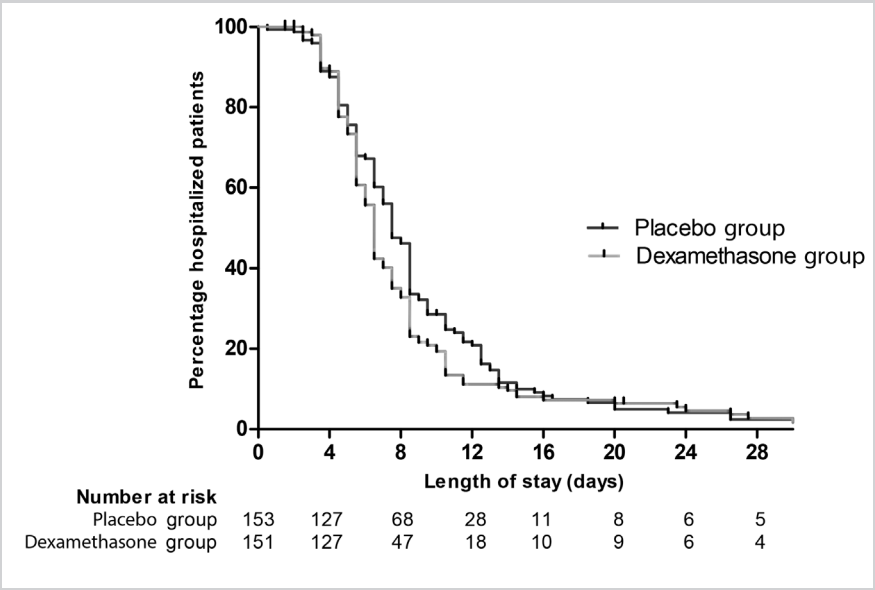

Figure 2. Kaplan-Meier analysis of the effect of dexamethasone on length of hospital stay in all enrolled patients. Patients who died or were admitted to the intensive care unit were censored on the day of death or the day of admission to the intensive care unit.

(Appendix F) and rates of admission to intensive care units did not differ between groups (Table 2). None of the patients received continuous positive airway pressure or non-invasive ventilation outside the intensive care unit. Rates of pleural effusion or empyema were less than 5% in both groups and did not differ significantly (p:0.54; Table 2). Seven (5%) patients in both groups were readmitted within 30 days of hospital discharge (Appendix G).

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

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

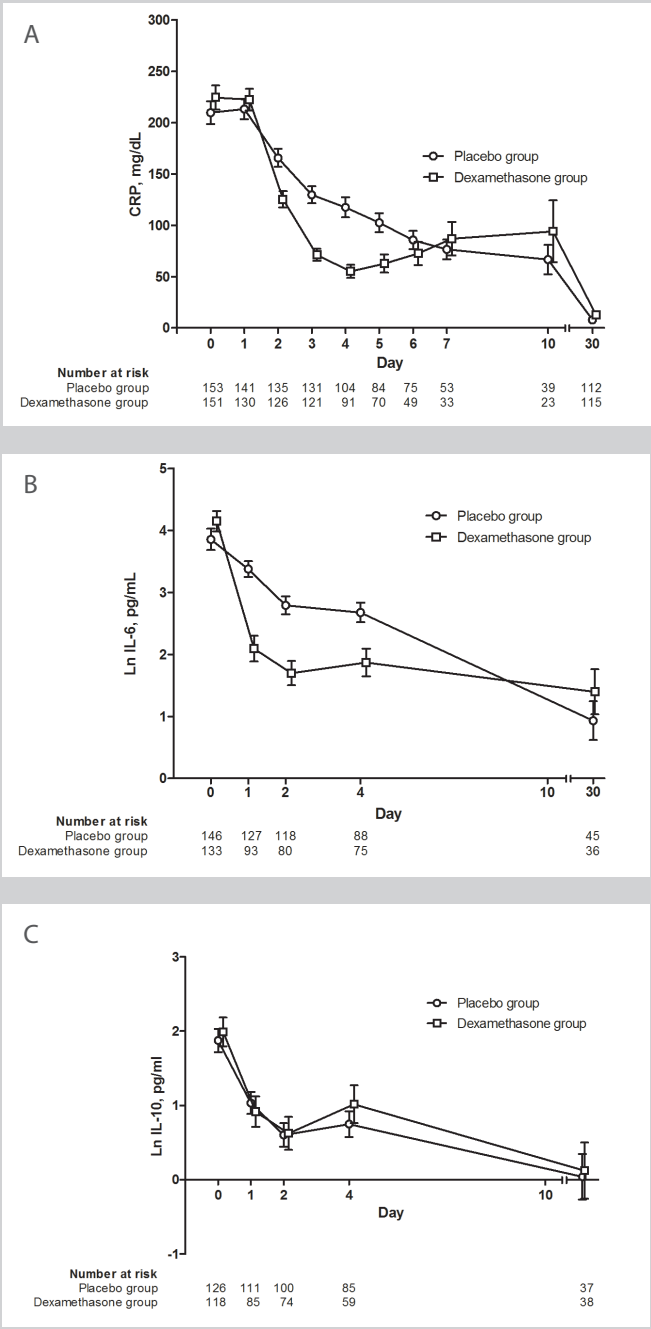

Figure 3. Mean concentrations of serum C-reactive protein (A), interleukin-6 (B), and interleukin-10 (C) from hospital admission to day 30. Error bars show standard error. Interleukin concentrations were not tested for all enrolled patients.

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

Hyperglycaemia (non-fasting glucose >11 mmol/L[17]) was more common in the dexamethasone group (67 [44%] patients) than it was in controls (35 [23%]; p<0.0001). However, only seven patients (5%) in the dexamethasone group and five patients (3%) in the placebo group needed additional glucose-lowering treatment during their hospital stay (p:0.57). Superinfection occurred in seven (5%) patients in the dexamethasone group and five (3%) patients in the placebo group (p:0.54). One patient in the dexamethasone group had a history of myelodysplastic syndrome, progressed to acute myeloid leukaemia on day 12 after admission, and subsequently died. Another patient in the dexamethasone group had a gastric perforation on day 3. Surgical closure of the perforation was done and the patient recovered well. Two patients in the placebo group developed an acute myocardial infarction on day 1; one died 4 days after admission to the intensive care unit and the other patient died after 3 weeks while on the ward. One patient in the placebo group required admission to the cardiac care unit because of new-onset atrial fibrillations. A masked independent monitoring committee (the Medical Ethics Committee, according to predefined regulations) adjudicated all adverse events and decided that there were no reasons for unmasking. Immunological and endocrinological data will be reported elsewhere.

Discussion

In our trial, we noted an overall reduction in median length of hospital stay of 1 day in patients with CAP who were given intravenous dexamethasone compared with controls. In a secondary analysis, patients in the dexamethasone group had a better quality of life than did controls with respect to social

functioning by day 30 after admission to hospital. These findings support our hypothesis that early administration of dexamethasone changes the immune response and thereby reduces length of hospital stay in patients with CAP. This modulation is shown in the accelerated return to normal concentrations of C-reactive protein and interleukin-6 that we noted in the dexamethasone group. However, interleukin-10 concentrations were not affected by the use of dexamethasone. The published effects of glucocorticosteroids on interleukin-10 concentrations during infection are variable,[18-20] and the effect of dexamethasone on interleukin-10 is probably dose-dependent.[21]

We reported an apparent rebound effect of dexamethasone on C-reactive protein concentrations by day 10 after admission to hospital, as previous described in the published work.[22] However, this finding might be explained because, by day 10, most patients in the dexamethasone group had been discharged, whereas the remaining patients had a complicated clinical course. By contrast, on day 10 the placebo group had a high number of patients who were almost ready for discharge and had low mean C-reactive protein concentrations. Moreover, the number of readmissions was not higher in the dexamethasone group than the control group, which would have been expected in the case of a true rebound effect.

Our results are in line with other studies that showed a beneficial effect of corticosteroids in patients with CAP. Confalonieri and colleagues reported an improvement in oxygenation and a survival advantage in patients with severe CAP who were treated with hydrocortisone for 7 days.[10] A retrospective study suggested that patients with severe CAP who were treated with systemic corticosteroids had a reduced risk of mortality compared with patients who were not given adjunctive corticosteroids.[23] A small randomised-controlled trial of 31 patients with CAP of any severity compared prednisolone for 3 days with placebo and reported a non-significant reduction in hospital stay from 16 to 11 days (p:0.182).[8] However, this study was probably too small to show significant effects on length of stay. A study of 213 patients — the largest so far to assess the role of prednisolone (40 mg once per day for 7 days) in CAP of any severity — showed neither beneficial effects of adjunctive corticosteroids on clinical cure at day 7 or effects on length of stay.[24] A possible explanation for the absence of effect compared with our study was the use of prednisolone once a day, which might not have been sufficient to achieve effective serum concentrations during the course of 24 h. Furthermore, this study was not powered to show differences in the length of hospital stay.

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

Our study has several strengths compared with previous studies. It was the largest randomised double-blind, placebo-controlled trial undertaken to date and was done in two hospitals. We used dexamethasone, which has a comparatively long biological half-life of 36–54 h.[25] Because we provided dexamethasone once a day for 4 days, the pharmacological effects can be expected from day 1 to about day 11. Moreover, because of the long half-life of dexamethasone, a more gradual reduction in biological effects might be expected, allowing for a gradual increase in intracellular glucocorticoid receptor number and recovery of the hypothalamic-pituitary-adrenal axis. Additionally, we measured total cortisol concentrations on the day of admission to detect adrenal insufficiency. The significance of a low serum cortisol concentration in patients with CAP is, however, not clear.[26] Nevertheless, in accordance with other studies, total cortisol concentrations of lower than 10 µg/dL were not associated with worse outcome than were higher concentrations.[27] Pneumonia severity index risk classes 4 and 5 were more commonly noted in the dexamethasone group than the placebo group. This imbalance in the severity of CAP could have led to an underestimation of the effect of dexamethasone because a high risk class (4 or 5) usually leads to a longer length of stay than does a low risk class (1–3).[28]

Our study had limitations. First, the results cannot be generalised to all patients with CAP. In patients with COPD, pneumonia is usually coincident with bronchial obstruction, which needs treatment with systemic corticosteroids[29] and therefore led to an underrepresentation of patients with COPD in this study (only 34 [11%] of 304 patients enrolled this study had COPD compared with an incidence of around 21% of the 817 people in the screened population). Also, the microorganism *C. burnetii* is somewhat overrepresented in this study because of an outbreak of Q fever in the Netherlands in spring 2009.[30] However, patients with *C. burnetii* pneumonia were equally distributed between the dexamethasone and placebo groups. Another limitation was that, because of low rates of antibiotic resistance, guidelines for antibiotic treatment in the Netherlands differ from US guidelines.[12] In the Netherlands, amoxicillin is standard therapy for CAP of pneumonia severity index class 1 and 2 and is combined with a fluoroquinolone or macrolide antibiotic in patients with more severe CAP. All pneumococci derived from sputum or blood cultures in

this study were sensitive to penicillin. A further limitation was that admission to intensive care units during hospital stay was defined as an endpoint of this study. Patients with severe CAP who were admitted to the intensive care unit were given corticosteroids according to the Surviving Sepsis Campaign protocol.[31] Therefore, we were unable to assess the effects of dexamethasone on mechanically ventilated patients. The study was not sufficiently powered to show an effect of dexamethasone on admission to the intensive care unit. Finally, dexamethasone was given intravenously. Although the study protocol allowed health-care professionals to stop the intravenous administration of dexamethasone if patients were switched to oral antibiotics, most patients received the full course of study medication. Therefore, participation in the trial might have resulted in longer administration of intravenous antibiotics. Although serious adverse events were rare, one patient in the dexamethasone group developed a gastric perforation on day 3 that could be attributed to the use of dexamethasone. Furthermore, hyperglycaemia was noted more often in the dexamethasone group than it was in the control group. Hyperglycaemia is also associated with adverse outcome in non-critically ill patients.[32] The benefits of corticosteroids should be weighed against the potential disadvantages of these drugs, such as superinfections and gastric disturbances.

Appendix A

Rand-36 assessment

The RAND-36 assesses physical and social functioning, physical and emotional role restriction, mental health, vitality, pain, general health and change in health in the 30 days preceding the assessment. The first questionnaire was taken on day three of admission and the second one was handed over to the patient 30 days after the day of admission to the hospital, i.e. at the moment of CRP measurement. Scales and items of RAND-36 range in score from 0 to 100. A high score represents a high level of quality of life and better functioning. A change of 10% of the scale breadth is perceptible to patients as a meaningful change, and a change in quality of life of 10 points is therefore considered clinically relevant.[33]

Appendix B

Pathogen identification

At least two sets of separate blood samples (drawn before the start of in-hospital antibiotic treatment) from each patient were cultured. Sputum specimens (if available) were Gram-stained and cultured; sputum samples with positive cultures were only used for further analysis if they fulfilled our definitions of representative sputum.[34] Urine antigen tests were performed for the detection of *Legionella pneumophila* serogroup 1 and *Streptococcus pneumoniae* (enzyme immunoassay, Binax-NOW; Binax, Portland, ME, USA). In-house developed polymerase chain reactions (PCR, Taqman real-time) were performed on the sputum to detect *Legionella spp., Mycoplasma pneumoniae, Coxiella burnetii* and *Chlamydophila psittaci*.[35] Paired serological testing was performed for the presence of antibodies to *Mycoplasma pneumoniae, Coxiella burnetii, Chlamydophila spp.* or respiratory viruses (adenovirus, influenza virus A and B, parainfluenza virus 1, 2 and 3 and the respiratory syncytial virus) (Serodia, Bipharma, Fujirebio Inc., Tokyo, Japan). A four-fold increase in antibody titre was considered positive. Pharyngeal samples were taken for viral culture or PCR ((para-) influenza virus, adenovirus and respiratory syncytial virus).

Appendix C

Antibiotic treatment in the two treatment groups.

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

Data are number (%).

Appendix D

Microbiological causes of pneumonia in the two treatment groups.

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

Data are number (%). Gram stain positive: *Rhodococcus equi*, *Group A streptococci*, *Group G streptococci*. Gram stain negative: *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Enterobacter cloacae*. Other viruses: adenovirus, parainfluenzavirus, respiratory syncytial virus, rhinovirus.

[1]: Mixed infection with influenza virus (n=4) or parainfluenza virus (n=3) or hMPV (n=1). [2]: Mixed infection with influenza virus (n=2) or parainfluenza virus (n=1). [3]: Mixed infection with *H. influenzae* (n=1). [4]: Mixed infection with parainfluenza virus (n=1). [5]: Mixed infection with influenza virus (n=2) or parainfluenza virus (n=1). [6]: Mixed infection with rhinovirus and parainfluenza virus (n=1). [7]: Mixed infection with influenza virus (n=1) or parainfluenza virus (n=1). [8]: Mixed infection with *S. pneumoniae* (n=1). [9]: Mixed infection with RS virus (n=1).

Appendix E

Reasons for protocol violation

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

Appendix F

Information on patients who died.

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

Appendix G

Reasons for readmission

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

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Dexamethasone downregulates the systemic cytokine response in patients with community-acquired pneumonia

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Abstract

Background

The influence of adjunctive corticosteroids on the cytokine response in community-acquired pneumonia (CAP) is largely unknown. In this study, we analysed the effect of dexamethasone on the cytokine response in patients with CAP and evaluated whether this effect is dependent on the causative microorganism. We hypothesised that dexamethasone has a larger effect on the cytokine response in patients with pneumococcal pneumonia than in patients with pneumonia caused by an atypical bacterium.

Methods

A total of 304 hospitalised, non-immunocompromised patients with CAP were randomised to an adjunctive four-day course of 5 mg dexamethasone once a day (n=151) or placebo (n=153). Serum concentrations of interleukin-1 receptor antagonist (IL-1ra), IL-6, IL-8, IL-10, IL-17, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), macrophage inflammatory protein-1α (MIP-1α) and monocyte chemotactic protein-1 (MCP-1) were measured on day 0, 1, 2, 4 and at control visit.

Results

Overall, the concentrations of IL-6 ($p<0.01$), IL-8 ($p<0.01$), MCP-1 ($p<0.01$) and TNF-α ($p<0.01$) were significantly lower on day 2 in the dexamethasone group than in the placebo group. In patients with pneumococcal pneumonia (n=72), both treatment groups showed a rapid decrease of cytokine concentrations; only the concentration of TNF-α ($p=0.05$) was significantly lower in the dexamethasone group on day 2. In patients with CAP caused by an atypical pathogen (*Legionella pneumophila, Chlamydophila* species, *Coxiella burnetii,* or *Mycoplasma pneumoniae;* total n=58), IL-1ra ($p<0.01$), IL-6 ($p<0.01$) and MCP-1 ($p=0.03$) decreased more rapidly in the dexamethasone group than in the placebo group.

Conclusions

Dexamethasone downregulates the cytokine response during CAP. This effect seems to be dependent on the causative microorganism. This study provides insight into which patients with CAP might benefit most from adjunctive dexamethasone.

Introduction

During a pulmonary infection, invading pathogens shed microbial components into the local environment. As a result, inflammatory cells become activated and will secrete a spectrum of cytokines and chemokines.[1] These cytokines and chemokines serve to control and eliminate the infection by leukocyte recruitment and inflammation. If not regulated tightly, the inflammatory response can become excessive and may progress into sepsis and ultimately multiple organ dysfunction syndrome (MODS). The nature and magnitude of the inflammatory response is determined by host characteristics, nature of causative microorganisms, as well as by antibiotic treatment.[2]

Glucocorticoids are potent physiological inhibitors of the inflammatory response. Currently, they are widely used as adjunctive treatment in various infectious diseases, such as meningitis and sepsis.[3-6] Recently, we have shown that adjunctive corticosteroids can also be beneficial in the treatment of community-acquired pneumonia (CAP): a four-day course of dexamethasone reduced the length of hospital stay by one day when added to antibiotic treatment in non-immunocompromised CAP patients.[7] Adjunctive therapy with corticosteroids might, hypothetically, downregulate excessive, potentially detrimental cytokine responses, and hereby accelerate clinical recovery.

Over the last decades, major advances have been made in the understanding of the molecular mechanisms by which glucocorticoids suppress inflammation. However, the influence of corticosteroids on the cytokine response in CAP is largely unknown. Up to now, only 2 studies have addressed this issue and they showed that corticosteroids can reduce the concentrations of interleukin-1β (IL-1β), IL-6 and tumour-necrosis-factor-α (TNF-α).[8, 9] Furthermore, whether the effect of dexamethasone on cytokines in CAP is dependent on the causative microorganism has never been investigated.

In this study, we analysed the effect of dexamethasone on the cytokine response in patients with CAP. Next, we evaluated whether the effect of dexamethasone on the cytokine kinetics depends on the causative microorganism of CAP. We hypothesised that dexamethasone has a larger effect on the cytokine response in patients with a pneumococcal pneumonia than in patients with pneumonia caused by an atypical bacterium, because pneumococci generally elicit a higher pro-inflammatory response in cases of pneumonia.[1]

Materials and methods

Patients and study design

This was a preplanned subanalysis of patients with CAP prospectively enrolled in a study on the effect of dexamethasone on length of hospital stay. The details of the study population and design have been described previously.[7] In short, from November 2007 until September 2010, adult patients with confirmed pneumonia were included at the emergency department of the St. Antonius Hospital in Nieuwegein or at the Gelderse Vallei Hospital in Ede, both hospitals in the Netherlands. Patients who were immunocompromised, on immunosuppressive therapy (including oral corticosteroids), or who required immediate admission to the intensive care unit (ICU) were excluded. All patients were randomised to either a four-day course of 5 mg (1 ml) of dexamethasone (dexamethasonedisodiumphosphate 5 mg, Centrafarm BV, Etten-Leur, the Netherlands) intravenously (IV) or 1 ml of sterile water (water for injection, Centrafarm BV, Etten-Leur, the Netherlands) IV. Randomisation was based on a one-to-one allocation by means of pre-numbered boxes containing four ampoules for IV administration. Patients, investigators and those assessing the data were masked to allocation. We calculated the Pneumonia Severity Index (PSI) score for all patients.[10] The local Ethics Committee approved the study and informed consent was obtained from all participants. (ClinicalTrials.gov number, NCT 00471640)

Analysis of the cytokine response

Serum was obtained on the day of presentation (before the first administration of dexamethasone) and subsequent samples were drawn at 8 A.M. on days 1, 2, 4, and at control visit at least 30 days after admission (convalescent phase). Serum samples were frozen at -80 °C until analysis. Circulating concentrations of IL-1ra, IL-6, IL-8, IL-10, IL-17, TNF-α, interferon-γ (IFN-γ), macrophage inflammatory protein-1α (MIP-1α) and monocyte chemotactic protein-1 (MCP-1) were measured by Milliplex multi-analyte profiling (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Data acquisition and analysis was performed on a Luminex 100 instrument (Luminex, Austin TX, USA).

Pathogen identification

At least two sets of separate blood and sputum samples (if available) from each patient were cultured. Urine antigen tests were performed for the detection of *Legionella pneumophila* serogroup 1 (Binax-Now; Binax, Portland, ME, USA)

and *Streptococcus pneumoniae* (Binax-Now; Binax, Portland, ME, USA). In-house developed polymerase chain reactions (PCR) were performed on the sputum to detect *L. pneumophila, Mycoplasma pneumoniae, Coxiella burnetii*, and *Chlamydophila psittaci*. Paired serological testing was performed for the presence of antibodies to *M. pneumoniae, C. burnetii, C. pneumoniae / psittaci* or respiratory viruses (adenovirus, influenzavirus A and B, parainfluenza virus 1, 2 and 3 and the respiratory syncytial virus) (Serodia, Bipharma, Fujirebio Inc, Tokyo, Japan). A fourfold increase in antibody titer was considered as positive. Pharyngeal samples were taken for viral culture ((para-)influenza virus, adenovirus and respiratory syncytial virus).

Statistical analysis

All statistical analyses were performed using statistics software (SPSS version 18.0 for Windows, Chicago, IL, USA). A two-tailed p-value of <0.05 was considered significant.

Differences in categorical variables were analysed with the Chi-square test or Fisher's exact test, and differences in continuous data were analysed with the Student's T-test. To investigate the influence of dexamethasone on the cytokine dynamics, linear regression analysis was performed. For the analyses, cytokine concentrations were transformed into a natural log scale, because of a non-normal distribution. We chose to analyse the decrease of the cytokines from day 0 to day 2, because all patients randomised to dexamethasone were at least 24 hours on dexamethasone on day 2 and because cytokine concentrations decreased most during the first days. To correct for the magnitude of the cytokine response on day 0, we included the cytokine concentration on day 0 as an independent variable in the linear regression.

To evaluate whether the effect of dexamethasone on the cytokine kinetics is dependent on the causative microorganism of CAP, we selected 3 aetiological subgroups: 1) patients with CAP caused by *S. pneumoniae,* 2) patients with CAP caused by an atypical pathogen, and 3) patients with CAP of unknown aetiology. Atypical pathogens include *L. pneumophila, M. pneumoniae, Chlamydophila* species and *C. burnetii.* Cytokine concentrations were compared between the three subgroups by linear regression analysis in a manner similar to that described above.

To examine the possible influence of antibiotic treatment on the cytokine response, a linear regression analysis, in which the variable 'appropriate antibiotic treatment' was added to the other variables ('randomisation' and the various cytokine concentrations on day 0) was performed.

Results

A total of 304 patients were enrolled in the study. The baseline characteristics of the patients are shown in Table 1. In 175 (58%) patients an aetiological diagnosis could be established. In 24% of the patients *S. pneumoniae* was detected, in 19% an atypical bacterium (*L. pneumophila,* 3.9%; *M. pneumoniae,* 1.6%; *C. burnetii*, 8.9%; *Chlamydophila* species, 4.6%), in 6% a Gram-negative bacterium other than *L. pneumophila*, in 6% a viral pathogen and in 2% another Gram-positive bacterium. There were no significant differences in aetiological diagnoses between the dexamethasone and the placebo group.

Table 1. Baseline characteristics of 304 patients with community-acquired pneumonia randomised to dexamethasone or placebo.

Characteristics	Dexamethasone group (n=151)	Placebo group (n=153)
Sex, male, no. (%)	84 (56)	87 (57)
Age, in years (SD)	64.5 (18.7)	62.8 (18.2)
Ethnicity*		
Caucasian, no. (%)	149 (99)	150 (98)
Other, no. (%)	2 (1.3)	3 (2.0)
Nursing home resident, no. (%)	9 (6.0)	7 (4.6)
Current smoker, no. (%)	38 (25)	38 (25)
Antibiotic treatment before admission, no. (%)	42 (28)	39 (25)
Comorbidities		
Neoplastic disease, no. (%)	9 (6.0)	10 (6.5)
Liver disease, no. (%)	2 (1.3)	0 (0.0)
Congestive heart failure, no. (%)	24 (16)	24 (16)
Renal disease, no. (%)	20 (13)	10 (7)
Diabetes mellitus, no. (%)	22 (15)	21 (14)
COPD, no. (%)	20 (13)	14 (9)
PSI Score (SD)	100.2 (33.4)	95.8 (32.5)
PSI risk class (no. of points), no. (%)		
I	18 (12)	22 (14)
II (≤ 70)	30 (20)	34 (22)
III (71-90)	24 (16)	33 (22)
IV (91-130)	54 (36)	43 (28)
V (> 130)	25 (17)	21 (14)
IV and V	79 (52)	64 (42)

Data are presented as number (%) or mean (SD).
*: Self-reported. Abbreviations: COPD, chronic obstructive lung disease; PSI, pneumonia severity index; SD, standard deviation

Dexamethasone reduces the magnitude of the cytokine response

During hospital stay, 151 patients received a four-day course of dexamethasone and 153 patients received placebo. Administration of dexamethasone changed the dynamics of the cytokine response (Figure 1). Cytokine concentrations were similar in both treatment groups on the day of hospital admission. The concentrations of IL-6 ($p<0.01$, β: -1.319 (-73%)), IL-8 ($p<0.01$, β: -0.423 (-35%)), MCP-1 ($p<0.01$, β: -0.385 (-32%)), TNF-α ($p<0.01$, β: -0.484 (-38%)) were significantly lower on day 2 in the dexamethasone treated patients, when compared to the placebo treated patients. IL-10 showed a rapid decrease in both treatment groups. Low systemic concentrations of IL-17 were found on admission and remained present during the study period (Figure 1).

Relation between the causative microorganism and the decrease in cytokine concentrations

To determine whether the influence of dexamethasone on the cytokine response is dependent on the causative microorganism, we compared the three selected aetiological subgroups of patients: patients with a pneumococcal pneumonia (n=72), patients with CAP due to an atypical microorganism (n=58) and patients with CAP of unknown aetiology (n=129).

In pneumococcal pneumonia, both treatment groups showed a rapid decrease of cytokine concentrations. Only the concentration of TNF-α ($p:0.05$, β: -0.227) was significantly lower in the dexamethasone group than in the placebo group on day 2 (Figure 2). In patients with CAP caused by an atypical pathogen, IL-1ra ($p<0.01$, β:-0.324 (-28%)), IL-6 ($p<0.01$, β:-0.400 (-33%)) and MCP-1 ($p:0.03$, β:-0.234 (-21%)) decreased more rapidly in the dexamethasone group (Figure 2). In patients with CAP of unknown aetiology, this effect of dexamethasone was seen for IL-6 ($p<0.01$, β:-0.485 (-38%)), IL-8 ($p<0.01$, β:-0.251 (-22%), TNF-α ($p<0.01$, β:-0.325 (-28%)) and MCP-1 ($p<0.01$, β:-0.307 (-26%). The cytokines and chemokines that did not show a significant influence of dexamethasone in CAP caused by either *S. pneumoniae* or an atypical pathogen are shown in Figure 3.

We next analysed whether the differences in effect on cytokine response between the dexamethasone and placebo group may be biased by appropriateness of empirical antibiotic treatment. This analysis was performed solely in patients with pneumonia caused by an atypical bacterium, because all patients with a pneumococcal pneumonia received the appropriate antibiotics upon admission. Analysis of the antibiotic prescriptions in patients with an atypical pathogen revealed that in the placebo group, 14 patients

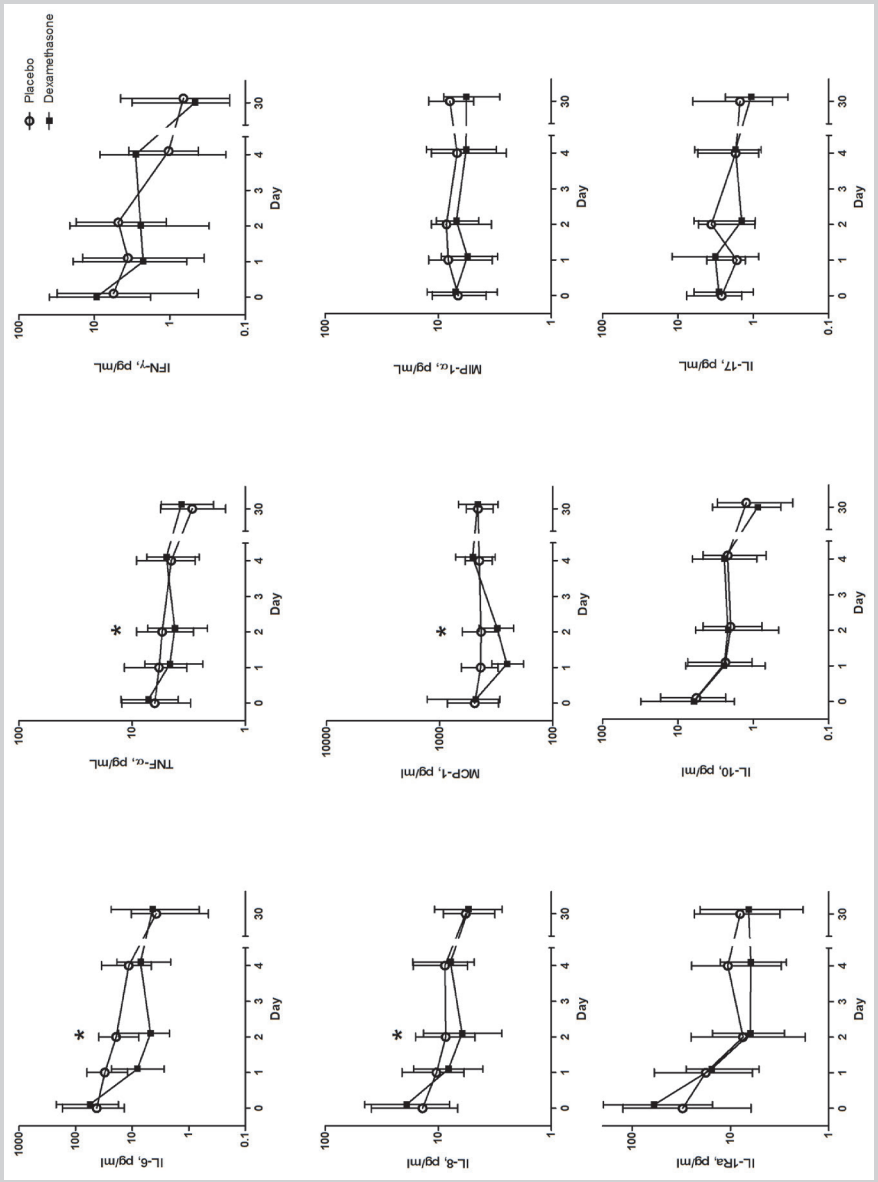

Figure 1. Median serum cytokine concentrations (with interquartile ranges) in patients with community-acquired pneumonia treated with either dexamethasone or placebo, from hospital admission to day 30. The asterisks indicate significant differences in the cytokine concentrations on day 2 between the placebo and dexamethasone groups (corrected for the magnitude of the cytokine response on day 0).

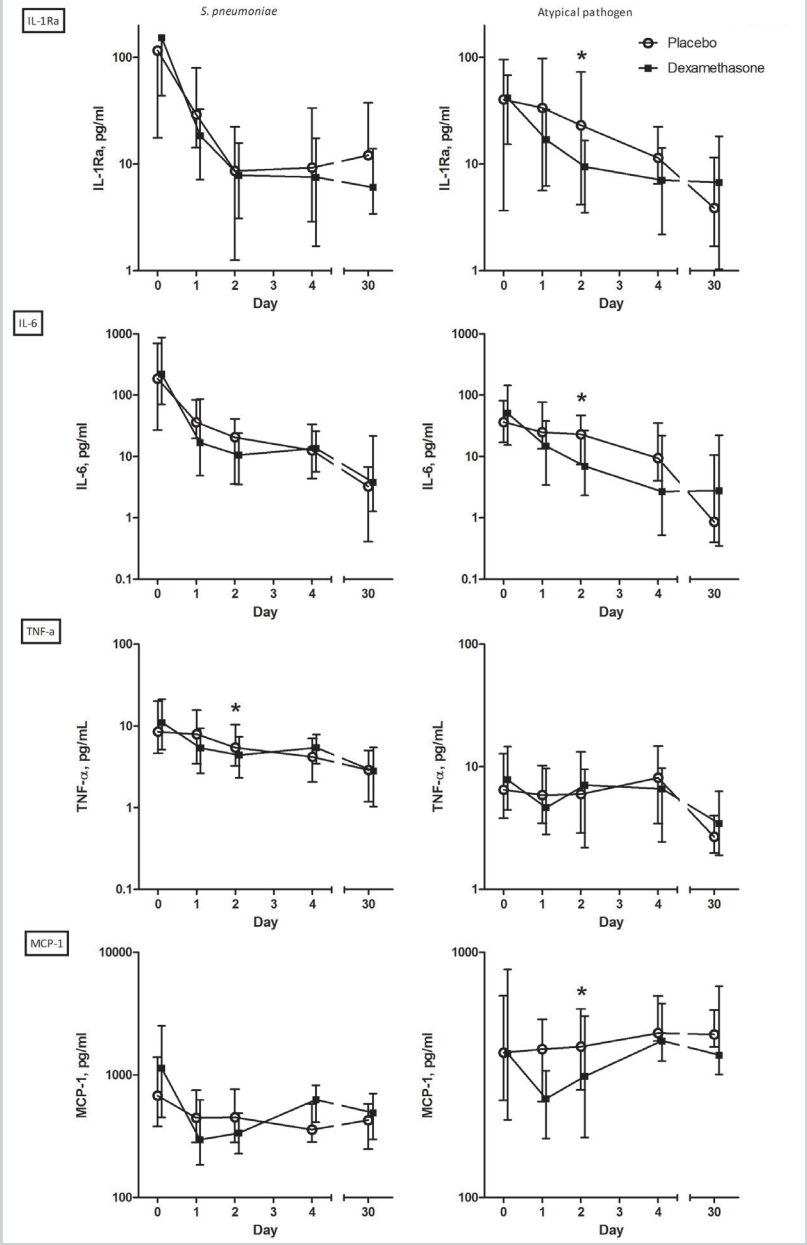

Figure 2. The influence of dexamethasone on median serum cytokine concentrations (with interquartile ranges) in patients with community-acquired pneumonia caused by either *Streptococcus pneumoniae* or an atypical bacterium, from hospital admission to day 30. This figure represents those cytokines that showed significantly different patterns in the two treatment groups, as indicated by an asterisk. Atypical bacteria include *L. pneumophila, M. pneumoniae, Chlamydophila* species and *C. burnetii*.

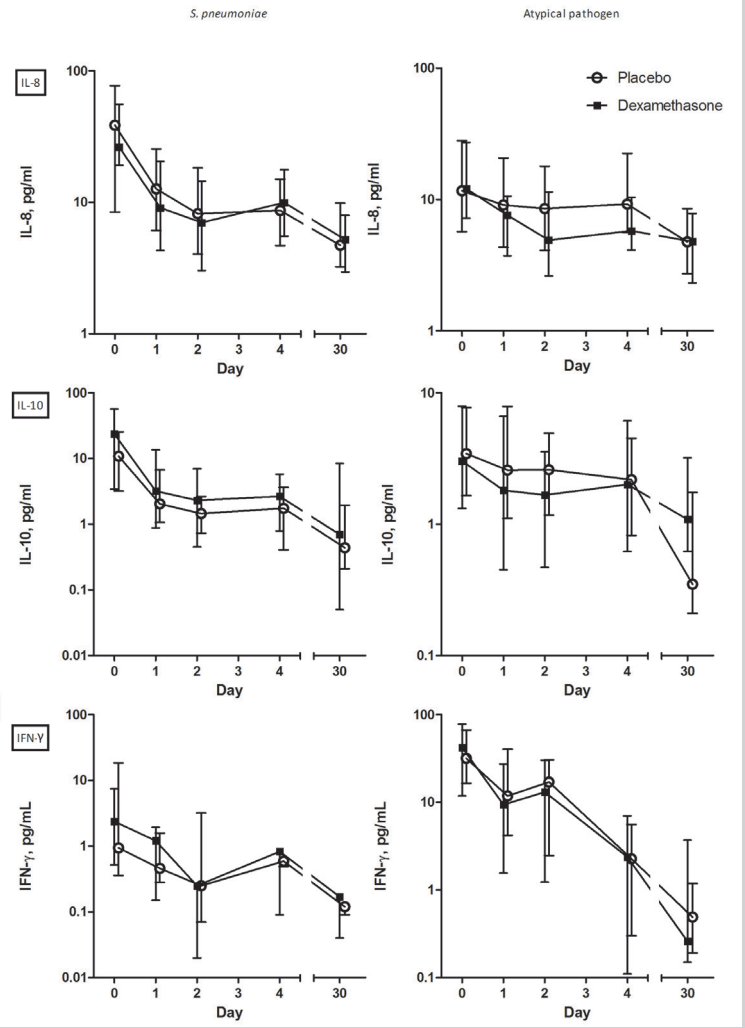

Figure 3. The influence of dexamethasone on median serum cytokine concentrations (with interquartile ranges) in patients with community-acquired pneumonia caused by either *Streptococcus pneumoniae* or an atypical bacterium, from hospital admission to day 30. This figure represents the cytokines that did not show different patterns in the two treatment groups. Atypical bacteria include *L. pneumophila*, *M. pneumoniae*, *Chlamydophila* species and *C. burnetii*. The concentration of IL-17 and MIP-1α in most cases fell out of range, below 3.2 pg/ml, which precluded reporting the course of these cytokines for the separate aetiological groups.

(44%) received the appropriate antibiotics on admission, while 18 patients (56%) were initially treated with an antibiotic that did not cover the causative microorganism. In the dexamethasone group, these figures were 13 patients (50%) for both groups (p:0.64). Linear regression revealed that only IL-6 concentrations were independently influenced by both randomisation ($p<0.01$, β:-0.330) and antibiotic treatment ($p<0.01$, β:-0.354). Thus, appropriateness of empirical antibiotic therapy had some impact on cytokine concentrations but could not fully explain the differences between the dexamethasone and placebo group. This confirmed that dexamethasone is capable of causing a considerable reduction of the cytokine concentrations.

Discussion

In this study we showed that, in general, adjunctive dexamethasone therapy reduces the concentrations of IL-6, IL-8, TNF-α and MCP-1 in patients with CAP. Interestingly, a clear difference in dexamethasone effects was found between different microbial aetiologies of CAP. Dexamethasone appeared to have little additional influence on the cytokine concentrations in patients with a pneumococcal pneumonia, while in patients with CAP caused by an atypical pathogen, dexamethasone gave a significantly faster decrease of cytokine concentrations than the placebo.

The overall dampening effect of dexamethasone on cytokine and chemokine concentrations is in concordance with the findings in other studies.[8, 9, 11-14] The majority of the former studies on corticosteroids in relation to cytokine responses have been performed in patients with septic shock.[11-14] To the best of our knowledge, only two studies have investigated this subject in patients with pneumonia.[8, 9] However, the generalisability of their results is limited, since in one study, all patients were mechanically ventilated, and in the study of Marik *et al*., only patients with severe pneumonia who were admitted to the ICU were evaluated. We are the first to report the influence of dexamethasone on a broader range of cytokines and in patients with CAP of all severities.

Prior CAP studies have not addressed the effect of dexamethasone on the cytokine kinetics in relation to the causative microorganism. In contrast with our hypothesis, we found little additional influence of dexamethasone in patients with pneumococcal pneumonia. The cytokine concentrations decreased rapidly during the first days of hospital admission in both the dexamethasone and the placebo group. This lack of an effect can possibly be explained by the high sensitivity to β-lactam antibiotics of *S. pneumoniae* strains in the Netherlands,

which might have had an overriding effect over any dexamethasone effect.[15] Interestingly, in the patients with CAP caused by an atypical bacterium, pro-inflammatory cytokines decreased more rapidly in the dexamethasone-treated patients. An additional analysis confirmed that the more prominent cytokine decrease in dexamethasone-treated patients was a true dexamethasone effect. Next to this dexamethasone effect, appropriateness of antibiotic treatment also played an independent role in the dynamics of IL-6.

A possible explanation for the variation in dexamethasone effects between various types of microorganisms is the difference in the cellular inflammatory response required for elimination of the pathogen. Atypical pathogens (most of them intracellular) require a mononuclear cell inflammatory response, compared to a more neutrophil-mediated response in typical (extracellular) bacterial microorganisms. This mononuclear cell inflammatory response stimulates a cytokine- and cell-mediated immune response. Corticosteroids particularly downregulate the cell-mediated immune response, and this might explain the more rapid decrease of cytokines in dexamethasone-treated patients with CAP caused by an atypical microorganism. In *M. pneumoniae* pneumonia, corticosteroid therapy in addition to antibiotics has already been advocated.[16, 17]

This study has some limitations. First, in the aetiological subgroup analysis, due to a lack of power, we were able to analyse only patients with pneumococcal pneumonia and a combined group of atypical microorganisms rather than analysing these pathogens separately. It is possible that there are even differences in cytokine response patterns among the atypical microorganisms, but larger studies are needed to allow statistical analysis of all pathogens separately. Second, only systemic cytokine measurements were performed in this study. The systemic cytokine response during CAP might differ from the local cytokine response in the lung. However, due to medical ethical restrictions, it was impossible to obtain bronchoalveolar lavage fluids of these patients during or after the active phase of the disease.

In conclusion, this study shows that cytokines are downregulated by adjunctive dexamethasone treatment. Our results suggest that the effect of dexamethasone on the cytokine response in CAP is dependent on the causative microorganism. This study provides insight in which patients with CAP might benefit most from adjunctive dexamethasone.

Larger studies are needed to further explore the exact role of the causative pathogen in the response to corticosteroids.

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

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Abstract

Background

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

Methods

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

Results

275 patients (131 dexamethasone, 144 placebo) were analysed. In the subgroup of patients (n=23) with a high cytokine response (IL-6≥92.5 pg/mL, IL-8≥14.8 pg/mL and MCP-1≥1154.5 pg/mL) and a discrepantly low cortisol (lowest 50%), dexamethasone treatment was associated with a significant decrease on a combined endpoint of mortality/ICU admission, as compared with placebo (0% vs. 43%, p<0.01). In the subgroup of patients with a high cytokine response and high cortisol (n=23), this favourable effect of dexamethasone was absent (30% vs. 39%, p:0.67).

Conclusions

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

Introduction

Community-acquired pneumonia (CAP) is a common disease that is still characterised by significant morbidity and mortality, despite adequate antibiotic treatment.[1] Last years, research has focused on adjuvant treatment strategies. A promising adjuvant therapy is the administration of corticosteroids during CAP. Corticosteroids are thought to exert their beneficial effect during inflammation by modulation of the immune response, or by relief of adrenal insufficiency.[2] Published studies, however, have shown results ranging from beneficial to no effect.[3-5]

Cortisol is an important regulator of inflammation. In general, the cortisol production increases during infection. Stress induced by severe illness strongly activates the hypothalamic-pituitary-adrenal (HPA) axis, which is an integral part of the host response to infection.[6] The increased cortisol level has several important effects on metabolism and the cardiovascular system, and exhibits anti-inflammatory and immunosuppressive effects. The latter are characterised by dampening of pro-inflammatory cytokines, chemokines and other inflammatory mediators. Furthermore, cortisol enhances the release of anti-inflammatory factors.[7]

Dysfunction of the HPA axis in critically ill patients, frequently referred to as critical illness-related corticosteroid insufficiency (CIRCI), has recently been defined as a random total cortisol of <10 µg/dL or a delta serum cortisol of <9 µg/dL after administration of 250 mcg synthetic adrenocorticotrophic hormone (ACTH, corticotropin test).[8] Critically ill patients lose their circadian rhythm in cortisol levels and therefore cortisol measurements in these patients can be performed at a random time of the day.[9]

Because cortisol is needed to control inflammation, an insufficient cortisol response during infection is expected to have an unfavourable impact on clinical outcome. This has been confirmed in patients with septic shock, in which CIRCI was associated with a higher risk of death.[10] Although not all studies have shown a beneficial effect of corticosteroids in these patients, therapy with low-dose corticosteroids is common in septic shock.[11, 12] Surprisingly, low cortisol levels (<10 µg/dL) were not associated with worse outcomes in patients with CAP.[13]

Furthermore, to date, no favourable effect of corticosteroids was found on mortality and intensive care unit (ICU) admission in the overall CAP population.[14] Recently, we have shown that dexamethasone can reduce the median length of hospital stay by 1 day when added to antibiotic treatment in patients with CAP.[5]

Whether there are specific subgroups of patients with CAP who especially might benefit from corticosteroid treatment is unknown. The correlation between systemic inflammation, as measured by cytokine response, and cortisol level has not been investigated thus far. Three of the most important pro-inflammatory cytokines during infection are interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemotactic protein-1 (MCP-1).[15-17] These cytokines have previously been found to correlate with disease severity or mortality in patients with CAP.[16-19] In this study, we aimed to investigate whether low cortisol levels are associated with adverse clinical outcome in CAP. Furthermore, we hypothesised that a discrepancy between systemic inflammation and cortisol level may identify a subgroup of patients who lack a sufficient cortisol response in CAP, and therefore particularly might benefit from dexamethasone administration.

Patients and methods

Patients

We performed a *post-hoc* analysis on data from patients with CAP enrolled in a study on the effect of dexamethasone on length of hospital stay. The details of the study population and design have been described previously.[5] In short, from November 2007 until September 2010 adult patients with confirmed pneumonia who were admitted to the St. Antonius Hospital in Nieuwegein or to the Gelderse Vallei Hospital in Ede, both teaching hospitals in the Netherlands, were prospectively enrolled in the study. In all cases, the attending physician on the emergency department decided that hospital admission was necessary. Diagnosis of pneumonia was confirmed when a new pulmonary infiltrate on a chest radiograph was present in combination with at least two of the following criteria: cough, sputum production, temperature more than 38^0C or lower than 35^0C, auscultatory findings consistent with pneumonia, C-reactive protein (CRP) concentration of more than 15 mg/L, white blood cell count of more than $10x10^9$ cells per L or fewer than $4x10^9$ cells per L, or more than 10% of rods in leukocyte differentiation. Patients who were immunocompromised, on immunosuppressive therapy (including oral corticosteroids), or who required immediate admission to the ICU were excluded. For the present analysis, patients using oral contraceptives or ketoconazole were excluded as well, because of their influence on serum cortisol levels. Furthermore, patients were not eligible when pneumonia was diagnosed more than 24 hours after admission. The need for ICU admission during hospitalisation and in-hospital mortality, as well as the length of hospital stay were assessed.

Study design

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

Laboratory tests

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

Statistical analysis

All statistical analyses were performed using SPSS 18.0 (Chicago, USA). A two-sided p-value of <0.05 was considered to be statistically significant. We show number (%) for categorical variables and median (IQR, interquartile range) for continuous variables with non-normal distribution or mean (SD, standard deviation) for those with normal distribution. Differences in categorical variables were analysed with the Chi-square test or Fisher's exact test, and differences in continuous data were analysed with Student's T-test or Mann-Whitney U-test, as appropriate.

Correlation analyses were performed by Spearman's rank correlation. To evaluate which patients benefitted most from dexamethasone therapy, all patients were classified based on the severity of systemic inflammation and total cortisol level. Severe systemic inflammation was defined as a high cytokine response on admission. Optimal cut-off points for IL-6, IL-8 and MCP-1 to predict the combined endpoint of mortality/ICU admission were determined using receiver operator characteristics (ROC) curve analysis and the Youden's index: IL-6 $\geq$92.5 pg/mL, IL-8 $\geq$14.8 pg/mL and MCP-1 $\geq$1154.5 pg/mL.[20] At first, all patients were divided into two subgroups: patients with all three cytokines above the cut-off values (high cytokine response) versus all other patients (low cytokine response). Subsequently, each group was further subdivided into "low" and "high" cortisol level, based on the median cortisol level of each group.

Finally, for all four subgroups, the effect of dexamethasone on a combined endpoint of mortality and ICU admission was evaluated. Additionally, we performed this analysis for the endpoint length of stay.

Results

Study population

A total of 304 patients were enrolled in this study. After the exclusion of 15 women who were using oral contraceptives and 14 patients missing a day 0 cortisol value, 275 patients were analysed. 131/275 patients (48%) were randomised to dexamethasone and 144/275 (52%) to placebo. The baseline patient characteristics are described in Table 1. There were no significant differences in median cortisol levels between patients with inhaled corticosteroids at home (n=25, cortisol 23.2 µg/dL, IQR 15.5–48.5) and without (n=250, cortisol 22.6 µg/dL, IQR 14.9–39.8) (p: 0.44).
During their hospital stay, 17/275 patients (6.2%) were admitted to the ICU, of which 4 patients deceased. In total, 16/275 patients (5.8%) died. On admission, 27/275 patients (9.8%) had a cortisol <10 µg/dL. Mean serum albumin did not differ between patients with cortisol <10 µg/dL and >10 µg/dL (p: 0.34). In patients with cortisol <10 µg/dL, 19/27 patients (70%) had a pneumonia that ranked as pneumonia severity index (PSI) class I–III, compared to 120/248 patients (48%) with cortisol >10 µg/dL (p: 0.03). None of the patients with cortisol <10 µg/dL died, compared to 16/248 patients (6.5%) with cortisol >10 µg/dL (p: 0.17). In the patients with cortisol <10 µg/dL, 2/27 patients (7.4%) were admitted to the ICU, compared to 15/248 patients (6.0%) with cortisol >10 µg/dL (p: 0.78).

Correlation between cytokine response and cortisol level on admission

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

Table 1. Baseline characteristics of 275 patients with CAP.

Characteristics	Placebo group (n=144)	Dexamethasone group (n=131)	p-value
Sex, no. of males (%)	84 (58.3)	79 (60.3)	0.74
Age, in years (SD)	63.9 (17.8)	66.5 (17.1)	0.22
Comorbidities			
Neoplastic disease (%)	10 (6.9)	9 (6.9)	0.98
Liver disease (%)	0 (0)	2 (1.5)	0.23
Congestive heart failure (%)	23 (16.0)	24 (18.3)	0.61
Renal disease (%)	10 (6.9)	19 (14.5)	0.04*
Diabetes mellitus (%)	21 (14.6)	20 (15.3)	0.87
COPD (%)	13 (9.0)	17 (13.0)	0.29
Pneumonia Severity Index score (SD)	90.0 (35.8)	97.6 (35.1)	0.08
Pneumonia Severity Index risk class			0.38
Class I (%)	18 (12.5)	11 (8.4)	
Class II (%)	31 (21.5)	23 (17.6)	
Class III (%)	31 (21.5)	23 (17.6)	
Class IV (%)	43 (29.9)	50 (38.2)	
Class V (%)	21 (14.6)	24 (18.3)	
Laboratory findings			
C-reactive protein, mg/L (SD)	212.1 (137.5)	223.3 (146.2)	0.51
Total cortisol, µg/dL (IQR)	223.1 (143.6-395.0)	236.4 (151.8-410.9)	0.73
Albumin, g/L (SD)	41.8 (7.4)	42.2 (8.3)	0.66
Outcome			
In-hospital mortality (%)	8 (5.6)	8 (6.1)	0.85
ICU admittance (%)	10 (6.9)	7 (5.3)	0.58
Mortality / ICU-admission (combined endpoint)	15 (10.4)	14 (10.7)	0.94

Data are presented as number (%), mean (SD) or median (IQR). COPD = chronic obstructive pulmonary disease; ICU = intensive care unit.
* Characteristics showing a significant association with a p-value <0.05

Cytokine response, cortisol level and the effect of dexamethasone

In total, 46/275 patients (17%) had a high cytokine response, indicating severe systemic inflammation. From these patients, 4/46 patients received inappropriate empirical antimicrobial therapy, of which 2 patients were assigned to placebo and 2 patients to dexamethasone. In 11 patients, macrolides were given as part of empirical combination therapy. Macrolide therapy was also equally balanced between both treatment groups. From all patients with a high cytokine response, 11/46 patients (24%) died or were admitted to the ICU, compared to 17/225 patients (7.6%) in the low cytokine response group ($p<0.01$).

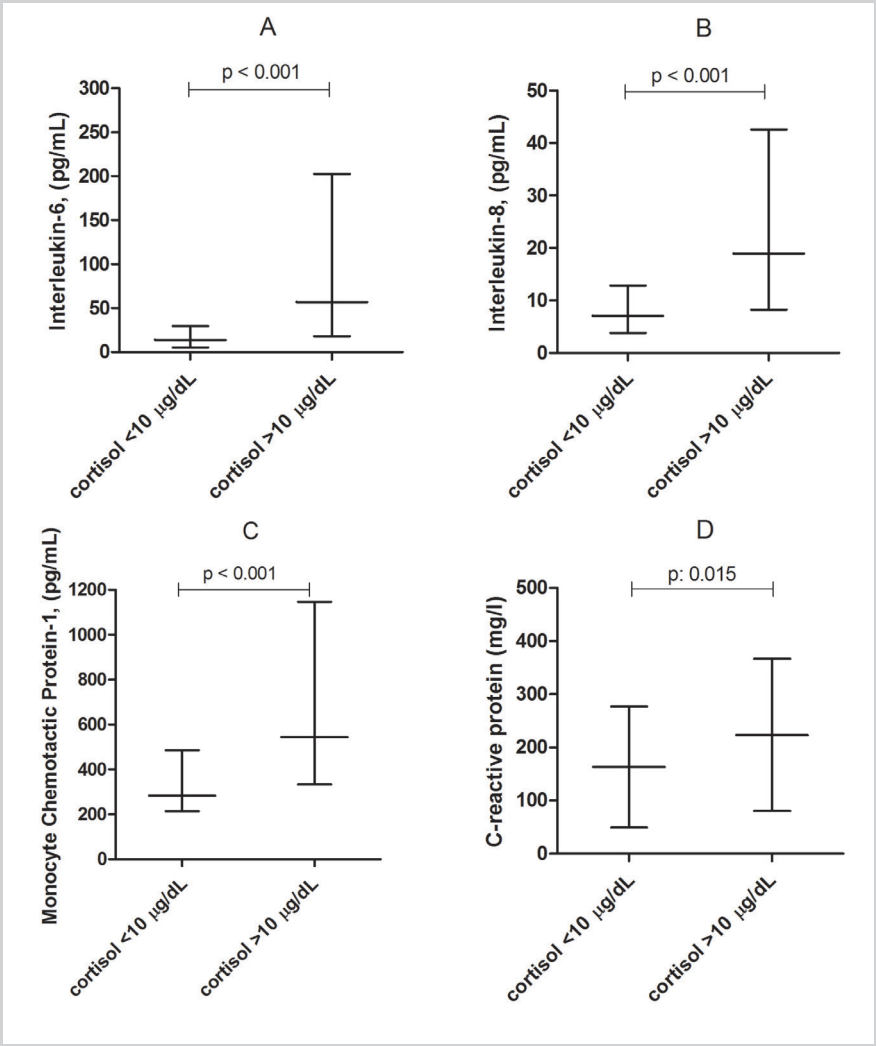

Figure 1. The correlation between cytokine response and cortisol level on admission. Comparison of the median levels and interquartile ranges of Interleukin-6 (panel A), Interleukin-8 (panel B), Monocyte Chemotactic Protein-1 (panel C) and the mean levels and standard deviations of C-reactive protein (panel D) between patients with cortisol <10 µg/dL and >10 µg/dL.

In patients with a high cytokine response, the median cortisol was 47.9 µg/dL (IQR 20.1–72.2). We evaluated whether patients with a high cytokine response and discrepantly low cortisol (<47.9 µg/dL) benefited more from dexamethasone therapy than patients with a high cytokine response and high cortisol (>47.9 µg/dL). The baseline characteristics of this subgroup of

interest were compared in Appendix A. As shown in Figure 2, in patients with a high cytokine response and discrepantly low cortisol (n=23), treatment with dexamethasone was associated with a significant decrease on a combined endpoint of mortality and ICU admission, compared with placebo (0/16 patients (0%) vs. 3/7 patients (43%), p<0.01). In patients with a high cytokine response and high cortisol (n=23), this favourable effect of dexamethasone on mortality/ICU admission was not found, as compared with placebo (30% vs. 39%, p: 0.67). Serum albumin did not differ between these two subgroups (41.4±8.2 g/L vs. 40.3±10.9 g/L) (p:0.70).

When we used the 25th percentile as a cut-off value for low cortisol (<20.1 µg/dL), the decrease on the combined endpoint of mortality/ICU admission caused by dexamethasone as compared to placebo was even higher, but the group was too small (n=12) to reach a significant effect (0% vs. 50%, p:0.091).

In patients with a low cytokine response (n=229), the median cortisol was 20.6 µg/dL (IQR 14.3-32.8). In contrast to patients with a high cytokine response, dexamethasone had no beneficial effect on mortality/ICU admission in patients with a low cytokine response and low cortisol (<20.6 µg/dL) (Figure 2).

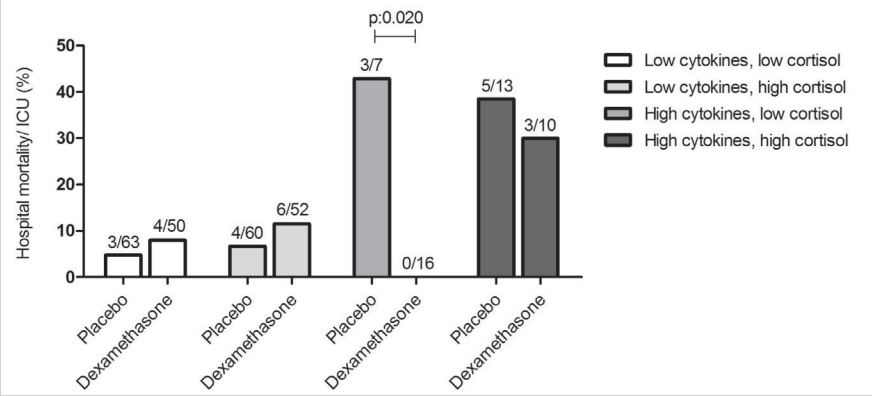

Figure 2. The cytokine response and cortisol level on admission predict the influence of dexamethasone on mortality/ICU admission in community-acquired pneumonia. All patients were classified into four groups, based on the combination of cytokine response (high or low) and cortisol level (high or low)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

Because a single parameter as indicator for the inflammatory response would be easier to use in clinical practice, we tried to replace the combination of three cytokines by CRP, a single cytokine or high PSI score (PSI class IV and V) on presentation. However, in those subgroups no significant clinical effect was found from dexamethasone treatment (data not shown).
Additionally, we investigated the effect of dexamethasone on median length of hospital stay. We observed a trend towards decreased length of stay for patients treated with dexamethasone in all subgroups, with the exception of patients with a high cytokine response and a high cortisol level, in which the median length of stay was almost equal (Appendix B).

Discussion

In this study, we confirm that a low serum cortisol <10 µg/dL on admission is not associated with an adverse outcome in patients with CAP. In a *post-hoc* subgroup analysis of patients with a high cytokine response and a discrepantly low cortisol level on admission (23/275 patients, 8.4% of the total study population), we showed, however, a beneficial effect of dexamethasone on a combined endpoint of hospital mortality and ICU admission.
Because cortisol is an important regulator of inflammation, an insufficient cortisol response during infection is expected to result in excessive, ongoing inflammation, which may be associated with a poor outcome. This is confirmed in patients with septic shock, in which CIRCI was associated with an adverse outcome.[10] However, in our study of patients with CAP, cortisol <10 µg/dL was not associated with adverse outcome. This is consistent with a recent meta-analysis of the existing literature regarding patients with CAP.[13] A possible explanation for the difference between septic shock and CAP might be that CAP patients with cortisol <10 µg/dL have a lower disease severity rather than corticosteroid insufficiency. Indeed, in our series, most of the patients with cortisol <10 µg/dL had a lower cytokine response and were more frequently classified in PSI classes I–III. In these cases, the low cortisol levels seem appropriate because of non-severe CAP.
The finding of a beneficial effect of dexamethasone in patients with a high cytokine response but a discrepantly low cortisol level suggests that these patients lack a sufficient adrenal response and are probably in the need of extra glucocorticoids to balance the intense pro-inflammatory response. Based on the present findings, the correlation between cytokine response and cortisol level might better reflect corticosteroid insufficiency during CAP and can help

to identify patients who will benefit most from dexamethasone treatment.
The mechanisms leading to dysfunction of the HPA axis during severe illness are complex and incompletely understood. Decreased production of the hormones corticotropin-releasing hormone (CRH), ACTH and cortisol, as well as tissue glucocorticoid resistance, may play a role in the development of adrenal insufficiency.[21] Corticosteroids potently inhibit inflammation by downregulating pro-inflammatory cytokine gene transcription.[22, 23] Besides this anti-inflammatory effect, the administration of synthetic corticosteroids can replenish the low tissue levels of endogenous cortisol during dysfunction of the HPA axis. Moreover, synthetic corticosteroids can (partially) overcome peripheral tissue glucocorticoid resistance.[24]
Our study has several strengths compared to previous studies. This is the first study that measures and correlates cytokine profiles and cortisol levels in patients with CAP. Due to the randomised controlled design of the study, we were able to assess the effect of a standardised dose of dexamethasone on outcome, compared with placebo.
Our study also had limitations. First, this is a *post-hoc* analysis of data generated in a recently published randomised controlled trial.[5] As a consequence, the numbers in the subgroups were small and in some subgroups the numbers of patients assigned to either placebo or dexamethasone were unequal. Therefore, the results of this study should be considered hypothesis-generating; a new prospective clinical trial must be conducted to confirm our findings. Second, due to the design of the study, only random total cortisol levels could be assessed. In the recent literature, CIRCI was defined as cortisol <10 µg/dL or delta serum cortisol <9 µg/dL after the administration of 250 mcg synthetic ACTH (corticotrophin test).[8] As corticotropin tests were not performed in our study, we were unable to identify patients with CIRCI, defined as delta serum cortisol <9 µg/dL after administration of 250 mcg synthetic ACTH. This did not influence our conclusion with respect to the absence of an adverse outcome in patients with cortisol <10 µg/dL. However, we might have missed an additional subgroup of patients with corticosteroid insufficiency that might also benefit from dexamethasone. Third, it is unclear whether the circadian rhythm of cortisol was lost in all patients. As a consequence, we might have found lower cortisol levels in some patients. Fourth, in our analysis to better define the patients who benefited most from dexamethasone treatment, we used the median cortisol level to discriminate between low and high cortisol. The number of patients was too small to allow proper analyses with other cortisol cut-off values.

Finally, the results cannot be generalised to all patients with CAP, because we excluded patients who were immunocompromised or needed immediate transfer to the ICU.

In conclusion, this *post-hoc* analysis confirmed that a total serum cortisol <10 µg/dL in patients with CAP is not associated with adverse outcomes. Most of these patients did have non-severe disease instead of corticosteroid insufficiency. We further observed that in a small subgroup of patients with severe systemic inflammation, as indicated by a high cytokine response, and a discrepantly low cortisol on admission, dexamethasone treatment was associated with a significant decrease on a combined endpoint of mortality and ICU admission. This correlation between cytokine response and cortisol level might better reflect corticosteroid insufficiency during CAP and can help to identify patients who will benefit most from dexamethasone treatment. Further prospective clinical studies are needed to confirm this hypothesis.

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We thank Mr. Ben de Jong, BSc, for his skilful technical assistance.

Appendix A

Baseline characteristics of 46 patients with a high cytokine response and a low cortisol level on admission with CAP.

Characteristics	Placebo group (n=7)	Dexamethasone group (n=16)	p-value
Sex, no. of males (%)	4 (57.1)	7 (43.8)	0.67
Age, in years (SD)	47.1 (26.4)	61.8 (16.7)	0.12
Comorbidities			
Neoplastic disease (%)	0 (0)	5 (31.3)	0.27
Liver disease (%)	0 (0)	0 (0)	
Congestive heart failure (%)	0 (0)	1 (6.3)	1.00
Renal disease (%)	0 (0)	4 (25.0)	0.27
Diabetes mellitus (%)	1 (14.3)	3 (18.8)	1.00
COPD (%)	0 (0)	4 (25.0)	0.27
Pneumonia Severity Index score (SD)	68.6 (31.7)	100.6 (36.6)	0.06
Laboratory findings			
C-reactive protein, mg/L (SD)	198.9 (179.0)	285.4 (178.1)	0.30
Total cortisol, µg/dL (IQR)	182.0 (121.9-397.9)	216.3 (178.4-339.6)	0.84
Albumin, g/L (SD)	40.7 (9.3)	41.7 (8.0)	0.79

Data are presented as number (%), mean (SD) or median (IQR). COPD = chronic obstructive pulmonary disease; ICU = intensive care unit.

Appendix B

The correlation of length of hospital stay with cytokine response and cortisol level.

Subgroup	Randomisation	Number of patients	Length of stay	p-value
High cytokines & high cortisol	Placebo	13	9.0 (5.0-14.5)	0.62
	Dexamethasone	10	9.5 (6.5-20.0)	
High cytokines & low cortisol	Placebo	7	12.0 (4.0-25.0)	0.59
	Dexamethasone	16	7.5 (6.3-10.8)	
Low cytokines & high cortisol	Placebo	60	9.0 (7.0-11.0)	0.07
	Dexamethasone	52	7.0 (6.0-9.8)	
Low cytokines & low cortisol	Placebo	63	7.0 (5.0-11.0)	0.26
	Dexamethasone	50	6.0 (4.8-9.0)	

Data on length of stay are presented as median (interquartile range)

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Changes in serum cortisol levels during community-acquired pneumonia: the influence of dexamethasone

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Abstract

Background

In community-acquired pneumonia (CAP), the cortisol level on admission can be a useful biomarker for prognosis. Serial cortisol measurements during the clinical course of disease and their association with disease outcome have never been reported. Furthermore, the time to recovery of the hypothalamic-pituitary-adrenal axis after a short course of dexamethasone during infection is unclear.

Methods

We analysed data from 270 hospitalised patients with CAP. Total serum cortisol was measured on presentation, day 1, 2, 4 and on control visit (day 30). Intensive care unit (ICU) admission and mortality were assessed. Additionally, to study the influence of dexamethasone on the kinetics of the cortisol response, we analysed serial cortisol values of 43 patients treated with a four-day regimen of dexamethasone 5 mg.

Results

During hospital stay, 26/270 patients (9.6%) were admitted to the ICU and 15/270 patients (5.6%) died. Compared to patients with an uneventful recovery, cortisol on presentation was significantly higher in patients with an adverse outcome (360 µg/L, IQR 209-597 vs. 238 µg/L, IQR 151-374) (p:0.01), and also remained significantly higher throughout the course of disease. Dexamethasone treatment resulted in nearly complete suppression of the endogenous cortisol production after the first dose, but cortisol production was fully recovered on control visit.

Conclusions

We show that an adverse outcome of CAP is associated with persisting higher total serum cortisol throughout the course of disease. Delta-cortisol could be another meaningful biomarker in CAP. Next, our data indicate that a four-day dexamethasone regimen during CAP does not lead to prolonged secondary adrenal insufficiency.

Main text

Cortisol, the predominant corticosteroid secreted by the adrenal cortex, is an important endogenous regulator of inflammation. During an infectious episode, cortisol production increases, and exerts anti-inflammatory and immunosuppressive activities.[1] In patients with community-acquired pneumonia (CAP), a high serum cortisol at the moment of hospital admission is associated with adverse outcome.[2] Therefore, cortisol could be a useful biomarker for prognosis in CAP. To our knowledge, serial cortisol measurements over the course of disease and their association with disease outcome have never been reported. Synthetic corticosteroids are attractive as adjuvant therapy in CAP, although conflicting data have been published. A potential risk of corticosteroids is secondary adrenal insufficiency.[3] The time to recovery of the hypothalamic-pituitary-adrenal (HPA) axis after a short course of dexamethasone during infection is unclear. For these reasons, we determined serum cortisol in patients with CAP on hospital admission and during the clinical course, until patients were recovered.

We analysed data from two clinical studies carried out in two teaching hospitals in the Netherlands. Both studies enrolled patients with confirmed pneumonia, using identical inclusion criteria. The study methods have been described previously.[4, 5] Patients who were immunocompromised, on immunosuppressive therapy (including oral corticosteroids), or who required immediate admission to the intensive care unit (ICU) were excluded. For the present study, patients using oral contraceptives, ketoconazole or patients receiving corticosteroids during hospital stay were also excluded. Additionally, we analysed 43 randomly selected patients receiving dexamethasone as part of a clinical trial to explore the effect of dexamethasone on cortisol. Total serum cortisol was measured on presentation, on day 1, 2, and 4 (at 8 A.M.), and on control visit on day 30 by immunoassay (Calbiotech, Spring Valley, USA). Statistical analyses were performed using SPSS 18.0 (Chicago, USA). A two-tailed p-value <0.05 was considered significant. Patients were excluded from the study after ICU admission. We measured differences in cortisol, albumin and C-reactive protein (CRP) with the Students T-test or the Mann-Whitney U test as appropriate. Logistic regression was used to assess possible confounding between variables. 270 patients were analysed (mean age 62.3±18.0 years; 60% male). The mean pneumonia severity index (PSI) score was 86.5±35.5 and 107/270 patients (40%) were classified as PSI class IV-V. Patients presented at the hospital at any

given moment of the day. The cortisol level on presentation therefore could be biased by circadian effects. However, during critical illness the circadian rhythm is expected to be overridden.[6] To determine whether the circadian rhythm of cortisol was lost on presentation, the time of cortisol measurement was plotted against the height of cortisol. Subsequently, the Kruskal-Wallis test was used to test for differences in height of cortisol. Indeed, we found no association between cortisol level and time of the day (p:0.51). Cortisol was higher in patients with inhaled corticosteroids at home (305 µg/L, IQR 176-527), compared to patients without inhaled corticosteroids at home (231 µg/L, IQR 153-374) (p:0.03).

During hospital stay, 26/270 patients (9.6%) were admitted to the ICU and 15/270 patients (5.6%) died. Cortisol on presentation was significantly higher in patients who died or were admitted to the ICU (n=34, 360 µg/L, IQR 209-597), compared to patients with an uneventful recovery (238 µg/L, IQR 151-374) (p:0.01). This was independent of the use of inhaled corticosteroids at home. Serum albumin did not differ significantly between both groups (41.1±9.3 g/L vs. 43.6±7.6 g/L) (p:0.09). In patients with an adverse outcome cortisol remained significantly higher throughout the course of disease, compared to patients with an uneventful recovery (Figure 1).

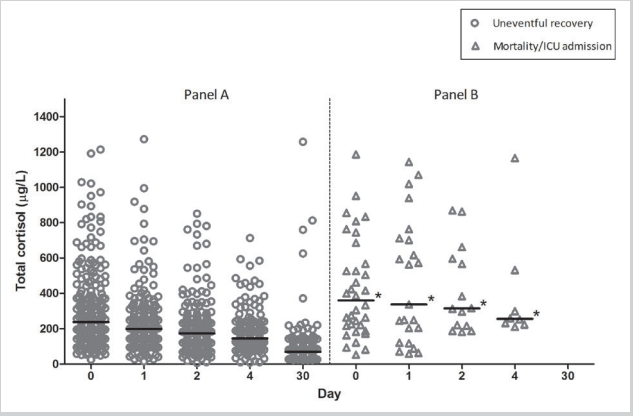

Figure 1. Changes in serum cortisol in hospitalised patients with community-acquired pneumonia. Total serum cortisol levels are shown for patients with an uneventful recovery (panel A) and for patients who died or were admitted to the ICU (panel B). Data points for individual patients are shown; horizontal bars indicate median values. Asterisks indicate a significant difference (p<0.05) between both groups on corresponding days.

Based on the association between the course of cortisol during CAP and clinical outcome, we hypothesized that the change in serum cortisol from day 0 to day 1, 2 or 4 (delta (Δ) cortisol 0-1, 0-2 or 0-4) could be a better prognostic biomarker in CAP. To assess the predictive value of Δ-cortisol, Receiver Operator Characteristics (ROC) curve analysis was performed. Δ-cortisol 0-4 appeared to be the best predictor for adverse clinical outcome (AUC 0.73, 95% CI 0.53–0.93), followed closely by Δ-cortisol 0-2 (AUC 0.72, 95% CI 0.56–0.89). The additional predictive value of Δ-cortisol to the initial cortisol level or other biomarkers or clinical scores was calculated using binary regression and ROC curve analysis. When Δ-cortisol 0-4 or Δ-cortisol 0-2 was added to the cortisol level on presentation, the prognostic accuracy improved substantially (Table 1). Moreover, the predictive value of other commonly used biomarkers and clinical scores in CAP also improved considerably when Δ-cortisol 0-4 or Δ-cortisol 0-2 was added (Table 1).

Table 1. Prediction of adverse clinical outcome (combined endpoint mortality/ICU admission) in community-acquired pneumonia: results from Receiver Operator Characteristics (ROC) curve analysis.

Parameter	Number of patients (total n=270)	AUC	95% CI
Cortisol*	266	0.63	0.53–0.74
Cortisol* & Δ-cortisol 0-1	226	0.68	0.54–0.83
Cortisol* & Δ-cortisol 0-2	218	0.81	0.71–0.91
Cortisol* & Δ-cortisol 0-4	186	0.88	0.81–0.94
CRP*	270	0.65	0.55–0.75
CRP* & Δ-cortisol 0-1	226	0.66	0.53-0.79
CRP* & Δ-cortisol 0-2	218	0.73	0.56–0.90
CRP* & Δ-cortisol 0-4	186	0.75	0.52–0.98
CURB-65*	205	0.66	0.55–0.76
CURB-65* & Δ-cortisol 0-1	173	0.71	0.60-0.83
CURB-65* & Δ-cortisol 0-2	161	0.76	0.59-0.92
CURB-65* & Δ-cortisol 0-4	142	0.70	0.51–0.88
PSI score*	270	0.74	0.64–0.84
PSI score* & Δ-cortisol 0-1	226	0.77	0.67-0.88
PSI score* & Δ-cortisol 0-2	218	0.80	0.67-0.93
PSI score* & Δ-cortisol 0-4	186	0.77	0.63–0.91

* Measured on the day of presentation. Abbreviations: AUC, area under the curve; CI, confidence interval; CRP, C-reactive protein; CURB-65, CURB-65 Severity Score for Community-Acquired Pneumonia; Δ, delta; PSI score, Pneumonia Severity Index score.

In order to study the influence of dexamethasone on the kinetics of the cortisol response, 43 randomly selected patients treated with dexamethasone were analysed in detail. These patients received a four-day course of dexamethasone 5 mg intravenously once a day during hospital stay, starting on the day of presentation. We analysed the decrease of cortisol from day 0 to day 2 because all patients were at least 24 hours on dexamethasone on day 2. As expected, dexamethasone treatment resulted in nearly complete suppression of endogenous cortisol production in all patients (mean decrease of 87%). Cortisol levels on day 30 (range 25-35) were within the normal physiological range, without apparent differences between the dexamethasone and the control group (69 µg/L, IQR 45-110 vs. 67 µg/L, IQR 44-106) (p:0.87). Both groups had low CRP concentrations on day 30 (5.9±8.1 mg/L vs. 7.4±12.8 mg/L) (p:0.61), indicating resolution of inflammation. These data indicate that a four-day dexamethasone regimen does not lead to prolonged secondary adrenal insufficiency.

We are the first to report serial total serum cortisol measurements during the course of disease in patients hospitalised with CAP, together with its predictive value. Our study showed that persisting higher cortisol throughout the course of CAP is associated with adverse clinical outcome. Although we have found that serial cortisol measurements can add prognostic value to other biomarkers and clinical scores for prognosis in CAP, its clinical relevance remains to be further substantiated. Δ-cortisol 0-2 or Δ-cortisol 0-4 as a biomarker has the disadvantage that the prediction of mortality/ICU admission will be delayed by 2-4 days. From that perspective, biomarkers and clinical scores that can be used on the day of presentation are to be preferred. However, Δ-cortisol might be helpful in patients without clinical improvement after the first days of treatment. No change, or even an increase in serum cortisol, is indicative of an unfavourable outcome and might help in the decision-making to switch or extend therapy.

Previous studies that addressed the influence of corticosteroids on the HPA axis were not performed in patients with CAP and various durations of therapy were combined for analysis, which makes extrapolation of the results to clinical practice difficult.[3, 7] In the present study, a fixed dexamethasone regimen was used, enabling us to determine the effects of short-term therapy more reliably.

Some limitations of our study must be named. First, our study has not been designed to study cortisol responses as a primary endpoint and therefore

cortisol was measured on day 0 and 30 at a random moment of the day. However, this did not influence our day 0 cortisol levels, because the circadian rhythm of cortisol was lost on presentation. On day 30, the time of cortisol measurement was similar for both groups. Second, recovery of the HPA axis as measured by total serum cortisol possibly paints an incomplete picture. Ideally, Synacthen tests should have been performed. Finally, patients were excluded when admitted to the ICU. We therefore could not analyse the complete course of cortisol in patients with an adverse outcome.

In conclusion, we showed that an adverse outcome of CAP was associated with persisting increased total serum cortisol levels throughout the course of disease. Delta-cortisol could be another meaningful biomarker in CAP. This finding may aid in the further development of cortisol as a biomarker for prognosis in CAP. Next, we found that adjuvant dexamethasone therapy almost completely suppressed the endogenous cortisol production after the first dose, but cortisol production was fully recovered on day 30. These findings are particularly relevant for clinical practice because adjuvant treatment strategies in CAP are emerging, and corticosteroids appear to be a promising candidate.

Acknowledgments

We thank Mr. Ben de Jong, BSc, for his skilful technical assistance.

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III

Immunomodulation by macrolides

Immunomodulatory effects of macrolides during community-acquired pneumonia: a literature review

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Abstract

Background

Macrolides are known to possess immunomodulatory properties next to their antimicrobial effects. These immunomodulatory activities have been proven beneficial in chronic pulmonary inflammatory diseases. Whether macrolides also exert favourable immunomodulatory effects during acute inflammation and therefore can act as adjuvant therapy in community-acquired pneumonia (CAP) is less clear.

Methods

We aimed to give an overview of the existing evidence from *in vitro* and *in vivo* studies on the immunomodulatory effects of macrolides during CAP. A comprehensive search in the PubMed/Medline and Embase database was performed. Two investigators independently examined the eligible literature. Studies that dealt with the effect of macrolides on the immune response, in terms of cytokine secretion and number or function of inflammatory and structural cells during acute inflammation, were included.

Results

A total of 27 studies were included of which 15 were *in vitro* studies, 9 *in vivo*, 2 both *in vivo* and *in vitro*, and 1 was in human subjects. Although the methods and experimental model systems used in these studies are very heterogeneous, macrolides in general tempered inflammation caused by viable and non-viable bacteria or their products. Cytokine secretion decreased, as well as inflammatory and structural cell activation and histological inflammatory signs. Not all data, however, are consistent and sometimes pro-inflammatory effects were found.

Conclusions

The available literature suggests that macrolides can temper the inflammatory response during CAP, independent of their antimicrobial activity. However, because the studies differ in methodology, no definite conclusions can be drawn.

Introduction

Despite effective antibiotic treatment and vaccination strategies, community-acquired pneumonia (CAP) still causes considerable morbidity and mortality. Lower respiratory tract infections are among the leading infectious causes of death in the developed world.[1] Complications associated with CAP, such as severe lung injury, multi-organ failure and shock, result from a complex interplay of the effects of microorganisms and their products on the host, and the inflammatory reaction mediated by the host immune system. Although an adequate inflammatory response is necessary for the clearance of microorganisms, excessive inflammation can lead to ongoing local and systemic damage.[2]

In order to improve the outcome of CAP, research has focussed on adjuvant therapy next to antibiotic treatment. These therapies are aimed at either the microorganism or the host and target improvement of bacterial opsonisation, improvement of effector mechanisms of the immune response and limitation of immunopathology. Promising treatment options in CAP include corticosteroids, macrolide antibiotics, statins, immunoglobulins, activated protein C, mannose-binding-lectin substitution therapy and Toll-like receptor antagonists.[3] Macrolides are known to possess immunomodulatory properties beyond their direct anti-bacterial activities.[4] The immunomodulatory effects of macrolides are beneficial in chronic pulmonary inflammatory syndromes, such as diffuse panbronchiolitis, cystic fibrosis, asthma and bronchiectasis. In these chronic diseases, the administration of macrolides is associated with a decrease in disease severity, length of hospital stay and mortality.[5] Whether macrolides also exert favourable immunomodulatory effects during acute inflammatory conditions, such as CAP, is less clear. Worldwide, controversy still exists regarding the best empiric antimicrobial regimen for CAP. Several retrospective clinical studies have shown survival benefits in patients with CAP treated with macrolides in combination with β-lactam antibiotics, compared to patients treated with β-lactam monotherapy.[6-11] However, due to the retrospective design of those studies, no definite conclusions can be drawn on whether administration of macrolides to all patients treated for CAP is beneficial. More insight is needed in the exact mechanisms of the immunomodulatory actions of macrolides during acute inflammation. The aim of this review is to give an overview of the existing evidence from *in vitro* and *in vivo* studies on the immunomodulatory effects of macrolides during acute inflammation caused by microorganisms commonly responsible for CAP. We focus on the effect of macrolides on cytokine secretion,

and on the number and function of inflammatory and structural cells of the respiratory tract.

A comprehensive search was performed in the PubMed/MEDLINE and Embase database. The search was limited to publications in the English language up to 22 August 2011. The following search terms were used: macrolides, azithromycin, erythromycin, clarithromycin, erythromycin estolate, erythromycin ethylsuccinate, ketolides, roxithromycin, cethromycin, telithromycin, immuno-modulation, inflammation, inflammation mediators, cytokines, *Haemophilus influenzae, Streptococcus pneumoniae, Staphylococcus aureus, Mycoplasma, Chlamydia, Legionella* and *Coxiella*. Two investigators independently screened the identified titles and abstracts. The full text of potentially relevant articles was examined. We searched the reference lists of retrieved studies for additional relevant reports. Studies that dealt with the effect of macrolides on the immune response were included. An effect on the immune response was defined as a change in cytokine secretion or a change in number or function of inflammatory cells and structural cells of the respiratory tract. The search results and exclusion criteria are summarised in Figure 1.

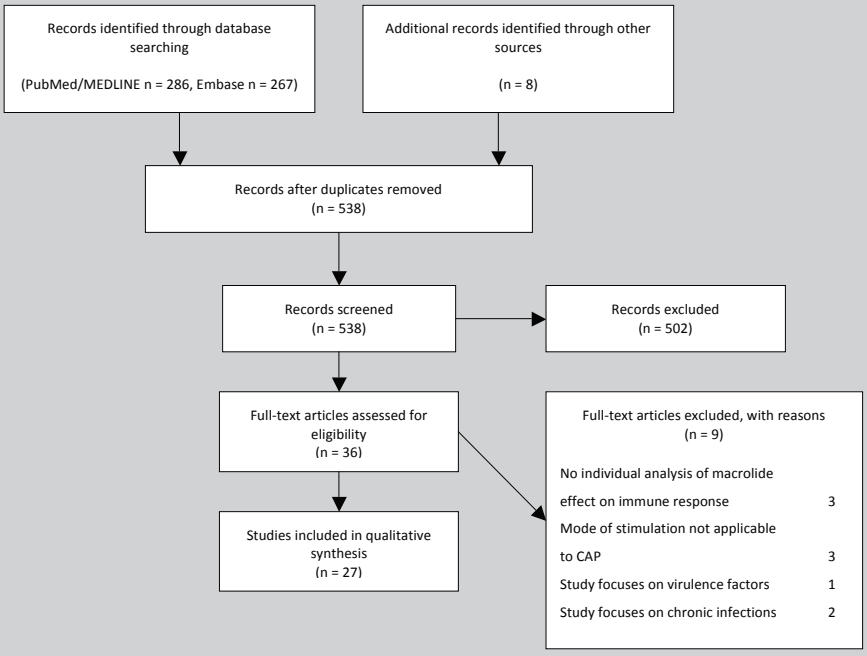

Figure 1. Search results and exclusion criteria

The initial search yielded 286 references for PubMed/MEDLINE and 267 references for Embase. Eight additional studies were identified through manual search. A total of 23 duplicates were removed. 502 studies were excluded based on title and abstract. Subsequently, 36 full-text articles were retrieved and screened. Eventually, we included and analysed 27 studies for this review (Figure 1).

The effects of macrolides on cytokine production

Cytokines are chemical messengers of the immune system that orchestrate the nature, intensity and duration of the immune response.[12] The major pro-inflammatory cytokines are interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), IL-6, and IL-12. Chemokines are a subgroup of cytokines that affect chemotaxis. Examples include IL-8 (CXCL8), of which keratinocyte chemoattractant (KC) is the murine homolog, macrophage inflammatory protein-1 (MIP-1; CCL3) and MIP-2 (CXCL2), monocyte chemoattractant protein-1 (MCP-1; CCL2), epithelial neutrophil-activating protein 78 (ENA-78; CXCL5) and regulated upon activation normal T-cell expressed and secreted (RANTES; CCL5). Anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist (IL1-ra), regulate the inflammatory response, for instance by inhibiting pro-inflammatory cytokine production or by counteracting the effects of pro-inflammatory cytokines.[13]

In vitro cytokine studies

Several *in vitro* studies evaluated the effect of macrolides on the cytokine production of endothelial, epithelial and inflammatory cells exposed to pathogens commonly causing CAP. In general, these studies showed that macrolides have a suppressive effect on cytokine secretion of several cell types (Table 1). This effect was found irrespective of whether viable[14, 15] or non-viable bacteria,[16-18] or bacterial products were used to stimulate the cells.[19-24] This strengthens the notion that tempering of the immune response takes place during macrolide treatment. Particularly interesting is the comparison of macrolide treatment of the cells with other antibiotics. For example, clarithromycin decreased IL-8 secretion by human alveolar type II cells when these cells were stimulated with crude antigens from *Mycoplasma pneumoniae*. This effect was absent when other antibiotics that are effective against *M. pneumoniae* (minocycline and ciprofloxacin) were used.[20] A decrease in cytokine secretion from epithelial cells was evident in case of infection with live bacteria after treatment with erythromycin but not after gentamicin.[15] Furthermore, cytokine secretion from whole blood and endothelium after

Table 1. Effects of macrolides on cytokine secretion *in vitro* sorted by cell type.

Cell type	Model	Macrolide	Pathogen/stimulation	Influence on cytokine secretion	No influence on cytokine secretion	Comments	Reference
nasal epithelium	human	ERY	*S. pneumoniae*	-	IL-8	-	Lagrou *et al.* (2000)[25]
nasal epithelium	human	ERY	LPS and IL-1β	-	IL-8	-	Lagrou *et al.* (2000)[25]
nasal epithelium	human	ERY	*M. pneumoniae*	-	IL-8, RANTES	there was no difference in cytokine secretion between infected and uninfected cells before ERY administration	Kazachkov *et al.* (2002)[26]
bronchial epithelium	human	ERY	*H. influenzae*, non typeable	↓IL-8, TNF-α	-	10mg/L ERY reduced cytokines compared to 0.1mg/L ERY or 100mg/L gentamicin	Qian *et al.* (2010)[15]
bronchial epithelium	human	ERY	*H. influenzae* endotoxin	↓ IL-6, IL-8	-	-	Khair *et al.* (1995)[19]
bronchial epithelium	human	ERY	IL-1β	↓IL-8	-	-	Khair *et al.* (1995)[19]
bronchial epithelium	human	CLR	*M. pneumoniae* membrane fraction	-	IL-8	MXF did not inhibit IL-8 either	Chmura *et al.* (2008)[28]
bronchial epithelium	human	CLR	TNF-α	↓IL-8	-	MXF inhibited IL-8 but only at the highest concentrations	Chmura *et al.* (2008)[28]
bronchial epithelium	human	AZM	*M. pneumoniae* membrane fraction	-	IL-8	MXF did not inhibit IL-8 either	Chmura *et al.* (2008)[28]
bronchial epithelium	human	AZM	TNF-α	↓IL-8	-	MXF inhibited IL-8 but only at the highest concentrations	Chmura *et al.* (2008)[28]
alveolar type II cells	human	CLR	*M. pneumoniae* antigen	↓IL-8	-	CIP and MIN did not affect cytokine secretion	Kurata *et al.* (2010)[20]

Table 1. *Continued I.*

Cell type	Model	Macrolide	Pathogen/stimulation	Influence on cytokine secretion	No influence on cytokine secretion	Comments	Reference
alveolar cells	murine	TEL	LPS stimulated macrophage supernatant	↓ MIP-2	-	-	Leiva *et al.* (2008)[23]
endothelium	human	ERY	*S. aureus* supernatant	↓IL-8, MCP-1	-	compared with β-lactams	van Langevelde *et al.* (1999)[22]
endothelium	human	CLR	*Chlamydia pneumoniae*	-	IL-8, MCP-1	-	Uriarte *et al.* (2002)[14]
endothelium	human	CLR	TNF-α	-	IL-8, MCP-1	-	Uriarte *et al.* (2002)[14]
endothelium	human	AZM	*C. pneumoniae*	↓IL-8*, MCP-1	-	-	Uriarte *et al.* (2002)[14]
endothelium	human	AZM	TNF-α	↓IL-8, MCP-1	-	-	Uriarte *et al.* (2002)[14]
endothelium	human	RXM	*C. pneumoniae*	↓IL-8	MCP-1	-	Uriarte *et al.* (2002)[14]
endothelium	human	RXM	TNF-α	↓IL-8	MCP-1	-	Uriarte *et al.* (2002)[14]
whole blood	human	ERY	*S. aureus* supernatant	↓IL-10, TNF-α	-	compared with β-lactams	van Langevelde *et al.* (1998)[21]
whole blood	human	ERY	*S. pneumoniae*, killed	↓ IL-6, TNF-α	IL-10, IL-12 p40, IL-12 p70, IFN-γ	-	Schultz *et al.* (1998)[16]
whole blood	human	ERY	*S. pneumoniae*, killed	↓ IL-6, IL-10, IL-12 p40, IL-12 p70, TNF-α, IFN-γ	-	compared to penicillin	Schultz *et al.* (1998)[16]

Table 1. *Continued II.*

Cell type	Model	Macrolide	Pathogen/stimulation	Influence on cytokine secretion	No influence on cytokine secretion	Comments	Reference
whole blood	human	ERY	*S. pneumoniae*, killed	↓IL-8, ENA-78	-	-	Schultz *et al.* (2000)[17]
whole blood	human	ERY	*S. pneumoniae*, killed	↓ IL-6, TNF-α	-	a decrease was evident with intravenous ERY and to a lesser extent with oral ERY	Guchelaar *et al.* (2001)[18]
PMNs	human	ERY	*S. pneumoniae* lysate	-	IL-8	short incubation time	Koch *et al.* (2000)[27]
PMNs	human	AZM	*S. pneumoniae* lysate	-	IL-8	short incubation time	Koch *et al.* (2000)[27]
PBMCs	human	ERY	TSST-1	↓ IL-2, TNF-α, IFN-γ	-		Kushiya *et al.* (2005)[24]
PBMCs	human	CLR	TSST-1	↓ IL-2, TNF-α, IFN-γ	-		Kushiya *et al.* (2005)[24]
PBMCs	human	AZM	TSST-1	↓ IL-2, TNF-α, IFN-γ	-	AZM inhibited more strongly than ERY and CLR	Kushiya *et al.* (2005)[24]
macrophages	murine	TEL	LPS	↓ TNF-α, MIP-2	-	TNF-α was also suppressed when LPS and TEL were added simultaneously	Leiva *et al.* (2008)[23]

↓ a significant decrease. AZM, azithromycin; CLR, clarithromycin; CIP, ciprofloxacin; ENA-78, epithelial neutrophil-activating protein 78; ERY, erythromycin; IFN, interferon; IL, interleukin; LPS, lipopolysaccharides; MCP, monocyte chemoattractant protein; MIN, minocycline; MIP, macrophage inflammatory protein; MXF; moxifloxacin; PBMCs, peripheral blood mononuclear cells; PMNs, polymorphonuclear leukocytes; RANTES, regulated upon activation normal T-cell expressed and secreted; RXM, roxithromycin; TEL, telithromycin; TNF, tumour necrosis factor; TSST, toxic shock syndrome toxin. *A downward trend was perceived but the decrease never reached significance.

stimulation with bacterial products was decreased during macrolide treatment in comparison with β-lactams.[21, 22] A similar effect was found when whole blood was stimulated with non-viable microorganisms.[16] In contrast with the above mentioned studies, in some publications macrolides did not influence cytokine secretion.[25-28] This lack of an effect could be due to a true absence of immunomodulatory effects, but might also be due to the methods employed in the experiments, such as the mode of stimulation of the cell, the cell types used or the incubation times. Overall, the results from *in vitro* studies suggest that macrolides have a suppressive effect on cytokine secretion in models of acute inflammation. However, because of the heterogeneity of the studies and the obvious difficulties in extrapolation of the results from *in vitro* to *in vivo*, these findings should be interpreted with caution.

In vivo cytokine studies

Next to *in vitro* experiments, several *in vivo* studies have investigated the effect of macrolides on cytokine secretion during acute inflammation. The findings of these studies are listed in detail in Table 2.

Ten studies examined the effect of macrolides on cytokine production during acute inflammation in murine pneumonia models. In five studies, macrolides decreased the concentration of cytokines and chemokines in bronchoalveolar lavage fluid (BALF) of mice infected with live bacteria.[29-33] In one of these experiments, cytokine secretion was decreased significantly while no effect on bacterial counts was found.[30] Two murine studies also found a decrease in secretion of cytokines when the infection was caused by macrolide resistant microorganisms.[34, 35] These data suggest that macrolides have immunomodulatory activities independent of their direct antibacterial effects. When acute inflammation was induced by killed microorganisms or bacterial products, however, conflicting results were found. Clarithromycin treatment of pneumonia induced with UV-killed *M. pneumoniae* caused no decline in cytokine secretion.[31] Surprisingly, BALF cytokine concentrations were even higher in clarithromycin treated animals. Similarly, no decline, but rather a rise in cytokine secretion was found when infection with *Mycoplasma* extract was treated with clarithromycin.[36] In contrast, the ketolide HMR 3004 did decrease cytokine secretion in animals inoculated with non-viable *S. pneumoniae*.[37] Another study evaluated a model in which pulmonary inflammation was induced by lipopolysaccharides (LPS). Here, treatment with telithromycin resulted in an attenuation of the cytokine response compared to untreated controls.[23] This illustrates that studies yielded very heterogeneous results when

Table 2. Effects of macrolides on cytokine secretion *in vivo* sorted by pathogen/stimulation.

Pathogen/stimulation	Model	Macrolide	Influence on cytokine secretion	No influence on cytokine secretion	Comments	Reference
S. pneumoniae	murine	HMR 3004	↓IL-6	-	compared with placebo	Duong *et al.* (2001)[32]
S. pneumoniae, macrolide resistant	murine	RXM	↓ KC ↑ MCP-1	IL-1β, TNF-α	compared with placebo	Yasuda *et al.* (2007)[35]
S. pneumoniae, killed	murine	HMR 3004	↓ IL-1β, IL-6	TNF-α	compared with placebo	Duong *et al.* (1998)[37]
M. pneumoniae	murine	CLR	↓IL-6, TNF-α, IFN-γ, KC, MCP-1, MIP1-α	IL-10	no significant difference in BAL bacterial cultures between placebo and CLR treated animals	Hardy *et al.* (2003)[31]
M. pneumoniae	murine	CLR	↓ IL-12 p40, KC, MCP-1, RANTES	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8 IL-9, IL-10, 1L-12p70, IL-13, IL-17, TNF-α, IFN-γ, MIP1-α, MIP1-β, G-CSF, GM-CSF, PDGF, VEGF, eotaxin	compared with placebo	Tagliabue *et al.* (2008)[33]
M. pneumoniae	murine	AZM	↓ IL-12, TNF-α, KC, MCP-1, MIP1-α	IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, GM-CSF	compared with placebo	Rios *et al.* (2005)[29]
M. pneumoniae	murine	CET	↓ IL-1β, IL-12, TNF-α, IFN-γ, KC, MCP-1, MIP1-α	IL-2, IL-4, GM-CSF	cytokines decreased whereas BAL cultures did not yet differ between CET group and placebo group	Rios *et al.* (2004)[30]
M. pneumoniae, killed	murine	CLR	↑IL-6	IL-10, TNF-α, IFN-γ, KC, MCP-1, MIP1-α	compared with placebo	Hardy *et al.* (2003)[31]

Table 2. *Continued.*

Pathogen/stimulation	Model	Macrolide	Influence on cytokine secretion	No influence on cytokine secretion	Comments	Reference
Mycoplasma extract	murine	CLR	↑IL-6, TNF-α, MCP-1, MIP-1α RANTES	IL-17, IL-23, KC	no increase in total cell and PMN counts in the lung despite higher cytokine concentrations in BALF	Hirao *et al.* (2011)[36]
H. influenzae, macrolide resistant	murine	CLR	↓ IL-1β, MIP-2	-	macrolide treatment reduced bacterial cultures despite resistance in high dose treatment group; cytokine levels were reduced in low dose treatment group despite no significant reduction in bacterial cultures	Nakamura *et al.* (2010)[34]
LPS	murine	TEL	↓ TNF-α, MIP-2	-	compared with placebo	Leiva *et al.* (2008)[23]
unknown	human	CLR	↓IL-6, ↑IL-10, IFN-γ	-	compared with amoxicillin	Demartini *et al.* (2004)[39]

↓ a significant decrease; ↑ a significant increase. AZM, azithromycin; BAL, bronchoalveolar lavage; CLR, clarithromycin; CET, cethromycin; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; IL, interleukin; IFN, interferon; KC, keratinocyte chemoattractant; LPS, lipopolysaccharides; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet derived growth factor; RANTES, regulated upon activation normal T-cell expressed and secreted; RXM, roxithromycin; TEL, telithromycin; TNF, tumour necrosis factor; VEGF, vascular endothelium growth factor.

different modes of stimulation and macrolides were used, especially in case of non-viable bacteria or bacterial products.

Although the studies in murine pneumonia models are very heterogeneous with regard to methodology and some of the results, there were several important consistent observations. In general, macrolides decreased cytokine levels in murine models of pneumonia induced with viable microorganisms. However, it is difficult to conclude whether this modulation of cytokine secretion is due to direct antimicrobial or to immunomodulatory effects of the macrolide in question. When macrolide-resistant microorganisms were used to infect the animals, still a decrease in cytokine levels was found. This suggests an immunomodulatory effect, independent of a direct antibacterial effect. However, macrolides are claimed to accumulate in phagocytic cells and epithelial lining fluid,[38] possibly causing drug concentrations to rise well above MIC. Nevertheless, in one study, the cytokine response already decreased while bacterial loads had not declined significantly, which would have been expected if the antimicrobial activity of macrolides was primarily responsible for the attenuation of the inflammatory response. This favours the concept of an immunomodulatory effect of macrolides. When pulmonary inflammation was induced with non-viable microorganisms, in some studies macrolides did not decrease cytokine secretion. This may mean that antimicrobial properties of macrolides predominate in the reduction of cytokine secretion. However, a decrease in cytokines was still found when acute lung inflammation was induced by LPS. A decrease would not be expected if only antimicrobial effects were exerted by macrolides. These findings together lead to the conclusion that the decline in cytokine levels found in murine models of pneumonia cannot be solely attributed to direct antimicrobial effects of macrolides, but must also be due to immunomodulatory activities.

One single *in vivo* study described the effect of macrolides on cytokine levels in human subjects with CAP. In this study, the effect of clarithromycin treatment was compared to amoxicillin. Treatment with clarithromycin resulted in a decrease in IL-6 and an increase in interferon-γ (IFN-γ) and IL-10 on the third and seventh day. In the patients treated with amoxicillin, no effect was found on IL-10, IFN-γ secretion decreased and a decline in IL-6 was evident only after seven days.[39] The proposed explanation by the authors is that this decrease was probably related to the resolution of inflammatory symptoms. This study suggests that clarithromycin can modulate cytokine production *in vivo* in humans with CAP. However due to the small sample size of the study and uncertainty about the methods employed, no definite conclusions can be drawn.

Although the decrease in cytokine secretion by macrolides is evident, it has to be kept in mind that extrapolation of *in vitro* as well as animal studies to clinical practice should be done with caution because to date there is no measure of inflammation by cytokines that has been calibrated. Well-designed clinical studies are required to correlate the degree of clinical inflammation with comprehensive analysis of cytokine patterns at the relevant body site.

The effects of macrolides on inflammatory cells

Polymorphonuclear leukocytes (PMNs) are the predominant cells that infiltrate the tissue in the early stages of an inflammatory response. During this time, chemokines activate PMNs which results in adhesion of these inflammatory cells to, for example, endothelium and subsequent transendothelial and transepithelial migration.[12] Activating signals stimulate metabolic pathways to generate a respiratory burst in PMNs, which produce reactive oxygen species and reactive nitrogen species. Although these substances play an important role in the killing of various microorganisms, they can also contribute to tissue damage in case of an excessive inflammatory response.[40]

In vitro studies on inflammatory cells

The effect of macrolides on inflammatory cells has been investigated in a number of *in vitro* studies, of which the results are depicted in Table 3. One study showed that PMNs that have been treated with azithromycin became committed to programmed cell death.[27] Incubation with erythromycin, penicillin or dexamethasone did not produce a similar increase in apoptosis. The addition of bacterial lysate of *S. pneumoniae* to PMNs inhibited the induction of apoptosis by azithromycin treatment.[27] Murine macrophages that were pretreated with telithromycin and subsequently challenged with LPS also showed increased apoptosis.[23] Azithromycin did not affect the oxidative function of PMNs when these cells were challenged with *S. pneumoniae.*[27] Both telithromycin and roxithromycin, however, completely arrested the oxidative metabolism in PMNs, whilst not affecting the bactericidal ability of these cells when stimulated with *S. aureus.*[41] In whole blood from healthy individuals treated with erythromycin *in vivo* and stimulated with heat killed *S. pneumoniae ex vivo*, enhanced degranulation of both azurophilic and specific granules in PMNs was found.[17] The combination of azithromycin with PMNs has been shown to augment the opsonophagocytic killing of *S. aureus* at concentrations above and below MIC.[42]

In vivo studies on inflammatory cells

Various murine pneumonia studies described the effect of macrolides on inflammatory cells *in vivo* (Table 3). Clarithromycin reduced both total cell counts and PMN counts in BALF in a murine model of *H. influenzae* pneumonia and pneumonia induced with *Mycoplasma* extract.[34, 36] In pneumonia caused by macrolide-resistant *S. pneumoniae*, a reduction in PMN cell infiltration in lungs was found in mice treated with roxithromycin, but compared to the control group, the lungs of the treated animals showed a greater influx of mononuclear phagocytes. Despite the reduction in PMNs, bacterial counts were lower in treated mice. This was attributed to the increase in mononuclear cells. Moreover, when mice were inoculated with killed microorganisms, the inflammatory response expressed by total cell counts in BALF was also reduced in treated animals.[35] HMR 3004 treatment of UV-killed *S. pneumoniae* pneumonia in mice resulted in lower PMN counts in BALF, compared to untreated mice. Moreover, there was an evident decline in nitric oxide (NO) release in the treatment group when compared to untreated animals.[37] HMR 3004 administration to viable *S. pneumoniae* pneumonia also resulted in a reduction of NO to levels comparable to those found in uninfected animals.[32] Telithromycin even decreased NO production in mice infected with LPS.[23] Moreover, lower total cell counts and PMN counts in BALF were found, compared with untreated mice.

From these studies it can be concluded that the behaviour of inflammatory cells changes to a more anti-inflammatory nature as a result of macrolide treatment. Inflammatory cells in murine pneumonia models that were treated with macrolides accumulated to a lesser degree in the affected lung compartments. This effect has been demonstrated in pneumonia caused by viable and non-viable bacteria or their products. These effects are promising for clinical practice because less accumulation of PMNs may result in less collateral damage of the lung. Another possible benefit is the finding of attenuation of NO release by PMNs after macrolide treatment, because excessive NO release can result in damage of lung tissue. Murine macrophages and human PMNs went into apoptosis when they were incubated in vitro with a macrolide. A balance tipping towards apoptosis rather than to necrosis of cells in case of acute inflammation can result in lower exposure of the immune system to cellular debris and thus limits the inflammatory response. Moreover, increased apoptosis of PMNs may also contribute to the reduction of PMN accumulation in the lung compartment. The pro-apoptotic effect of macrolides on host cells is possibly counteracted by the release of bacterial products. However, this dual effect in macrolide

Table 3. Effects of macrolides on inflammatory cells sorted by pathogen/stimulation.

Pathogen/stimulation	Model	Macrolide	Influence on inflammatory cells	Comments	Reference
none	*in vitro*	ERY	↑ apoptosis of PMNs without addition of *S. pneumoniae* lysate	addition of *S. pneumoniae* lysate abrogated apoptosis; PEN, ERY or DEXA did not induce apoptosis	Koch *et al.* (2000)[27]
S. pneumoniae	*in vitro*	AZM	no effect on oxidative burst PMNs	short incubation time	Koch *et al.* (2000)[27]
S. pneumoniae	*in vivo*	HMR 3004	↓ MPO activity (PMNs) in lung tissue ↓ monocytes, total cell counts and NO in BALF	compared with untreated controls	Duong *et al.* (2001)[32]
S. pneumoniae, macrolide resistant	*in vivo*	RXM	↓ MPO activity (PMNs) in lung tissue	compared with untreated controls; mononuclear phagocytes predominated in treated animals	Yasuda *et al.* (2007)[35]
S. pneumoniae, killed	*in vitro*	ERY	↑ degranulation of specific and azurophilic granules in PMNs	compared with situation before infusion of antibiotics	Schultz *et al.* (2000)[17]
S. pneumoniae, killed	*in vivo*	RXM	↓ total cell counts and PMN counts in BALF	compared with untreated controls	Yasuda *et al.* (2007)[35]
S. pneumoniae, killed	*in vivo*	HMR 3004	↓ MPO activity (PMNs) in lung tissue ↓ total cell counts and NO in BALF	compared with untreated controls	Duong *et al.* (1998)[37]
H. influenzae, macrolide resistant	*in vivo*	CLR	↓ total cell counts and PMN counts in BALF	compared with untreated controls	Nakamura *et al.* (2010)[34]
H. influenzae, endotoxin	*in vitro*	ERY	↓ chemoattraction of PMNs to bronchial epithelium	compared with untreated controls	Khair *et al.* (1995)[19]
Mycoplasma extract	*in vivo*	CLR	↓ total cell counts and PMN counts BALF	a decrease in cells was evident despite an increase in cytokine secretion; compared with vehicle treated controls	Hirao *et al.* (2011)[36]

Table 3. *Continued.*

Pathogen/stimulation	*Model*	Macrolide	Influence on inflammatory cells	Comments	Reference
S. aureus	*in vitro*	AZM	↑ opsonophagocytic quality of PMNs	compared to RP7293, diacetyl-midecamycin and ERY	Herrera-Insua *et al.* (1997)[42]
S. aureus	*in vitro*	RXM	↓ oxidative burst	bactericidal activity was unaffected despite attenuation of the oxidative burst	Vazifeh *et al.* (2002)[41]
S. aureus	*in vitro*	TEL	↓ oxidative burst	bactericidal activity was unaffected despite attenuation of the oxidative burst	Vazifeh *et al.* (2002)[41]
LPS	*in vivo*	TEL	↓ total cell counts, PMN counts and NO in BALF	compared with untreated controls	Leiva *et al.* (2008)[23]
LPS	*in vitro*	TEL	↑ apoptosis of murine macrophages	compared with untreated controls	Leiva *et al.* (2008)[23]

↓ a significant decrease.; ↑ a significant increase. AZM, azithromycin; BALF, bronchoalveolar lavage fluid; CLR, clarithromycin; DEXA, dexamethasone; ERY, erythromycin; LPS, lipopolysaccharides; MPO, myeloperoxidase; NO, nitric oxide; PEN, penicillin; PMNs, polymorphonuclear leukocytes; RXM, roxithromycin; TEL, telithromycin.

action, namely priming for apoptosis of PMNs in absence of bacterial products and absence of apoptosis when bacterial products are present, has not been sufficiently studied to draw conclusions on its mechanism or relevance for clinical practice. The observed enhancement of the opsonophagocytic activity of PMNs and the degranulation of azurophilic and specific granules in these cells by macrolides may result in more efficient elimination of the invading microorganism in CAP and hence a shorter period of inflammation.

The effects of macrolides on structural cells of the respiratory tract

Studies reporting the effects of macrolides on structural cells of the respiratory tract focus mainly on expression of adhesion molecules on endothelium and epithelium that aid in migration of inflammatory cells, the integrity of endothelium and the histological architecture of the lung.

Endothelium and epithelium

Various *in vitro* studies have demonstrated that macrolides affect structural lung cells on a cellular level (Table 4). In endothelium pre-treated with azithromycin and roxithromycin, and subsequently infected with *Chlamydia* or stimulated with TNF-α, significantly decreased transendothelial migration of PMNs and monocytes was found. In endothelium treated with clarithromycin this effect was absent.[14] Bronchial epithelium that was treated with erythromycin after *H. influenzae* endotoxin challenge expressed less soluble intracellular adhesion molecules (s-ICAM), that normally would aid in PMN adherence to epithelial cells. In addition when human endothelium was incubated with bronchial epithelium that was challenged with *H. influenzae* endotoxin and treated with erythromycin, a decrease in PMN adherence to endothelium was observed.[19] ICAM-1 expression of bronchial cells also decreased when nontypeable *H. influenzae* was treated with erythromycin.[15] ICAM-1 expression on human endothelial cells decreased when stimulated with supernatants from *S. aureus* treated with erythromycin, but not when treated with β-lactams. Erythromycin treatment also reduced PMN adhesion to endothelium, when compared to β-lactam treatment.[22] Furthermore, apoptotic activity was increased in murine alveolar type II cells that were pre-treated with telithromycin and afterwards incubated with supernatants from LPS-challenged macrophages.[23]

Table 4. The effect of macrolides on structural cells *in vitro* sorted by cell type.

Cell type	Model	Macrolide	Pathogen/stimulation	Influence on structural cells	Comments	Reference
endothelium	human	CLR	*C. pneumoniae*	no effect on PMN and monocyte TEM	compared with antibiotic free controls	Uriarte *et al.* (2002)[14]
endothelium	human	CLR	TNF-α	no effect on PMN and monocyte TEM	compared with antibiotic free controls	Uriarte *et al.* (2002)[14]
endothelium	human	AZM	*C. pneumoniae*	significant decrease in PMN and monocyte TEM	compared with antibiotic free controls	Uriarte *et al.* (2002)[14]
endothelium	human	AZM	TNF-α	significant decrease in PMN and monocyte TEM	compared with antibiotic free controls	Uriarte *et al.* (2002)[14]
endothelium	human	RXM	*C. pneumoniae*	significant decrease in PMN and monocyte TEM	compared with antibiotic free controls	Uriarte *et al.* (2002)[14]
endothelium	human	RXM	TNF-α	significant decrease in PMN and monocyte TEM	compared with antibiotic free controls	Uriarte *et al.* (2002)[14]
endothelium	human	ERY	*S. aureus* supernatant	reduced adherence of granulocytes to endothelium	compared with β-lactams; the effect is probably the result of a change in endothelium rather than inflammatory cells	van Langevelde *et al.* (1999)[22]
endothelium	human	ERY	*S. aureus* supernatant	reduced expression of ICAM-1 by endothelial cells	compared with β-lactams	van Langevelde *et al.* (1999)[22]
bronchial epithelium	human	ERY	*H. influenzae*, non typeable	decreased ICAM-1 expression	10mg/L ERY reduced ICAM-1 compared with 0.1mg/L ERY or 100mg/L gentamicin	Qian *et al.* (2010)[15]
bronchial epithelium	human	ERY	*H. influenzae* endotoxin	decreased s-ICAM-1 expression	compared with antibiotic free controls	Khair *et al.* (1995)[19]
alveolar cells type II cells	murine	TEL	LPS stimulated macrophage supernatant	increase in apoptotic activity	compared with antibiotic free controls	Leiva *et al.* (2008)[23]

AZM, azithromycin; CLR, clarithromycin; ERY, erythromycin; LPS, lipopolysaccharides; PMN, polymorphonuclear leukocytes; RXM, roxithromycin; (s)-ICAM-1, (soluble) intracellular adhesion molecule; TEL, telithromycin; TEM, transendothelial migration; TNF, tumour necrosis factor.

Histological architecture of the lung

Several studies evaluated the effects of macrolides on histological signs of inflammation in murine models (Table 5). In these studies, the degree of lung inflammatory changes is classified using a uniform scoring system that assigns points to visual parameters of inflammation (lung histopathologic score, HPS) or using the individual assessment of a pathologist.

Conflicting results were found for infection with *Mycoplasma* or its components. Clarithromycin, cethromycin and azithromycin therapy significantly reduced lung HPS in pneumonia caused by viable *M. pneumoniae*.[29-31, 33] In another study, clarithromycin, but not minocycline or ciprofloxacin, decreased pulmonary inflammation induced with crude antigens of *M. pneumoniae*.[20] These results suggest that macrolides exert local immunomodulatory effects next to antibacterial effects. However, in another model, clarithromycin did not affect inflammation in pneumonia induced by a clarithromycin-resistant strain of *M. pneumoniae*.[20] Also, treatment of pneumonia induced by non-viable *M. pneumoniae* with clarithromycin did not reduce HPS.[31] The proposed explanation for this is that certain bacterial strains may cause inflammation, which cannot be controlled by a macrolide.

Studies investigating the macrolide treatment of infection caused by microorganisms other than *M. pneumoniae* did yield consistent results. Roxithromycin treatment of murine pneumonia induced with macrolide-resistant *S. pneumoniae* and clarithromycin treatment of macrolide-resistant *H. influenzae* pneumonia, resulted in less damage to lung tissue as compared with controls.[34, 35] Also, the ketolide HMR 3004 decreased lung oedema in a model of pneumonia caused by UV-killed *S. pneumoniae* to a level that was comparable to that in the uninfected control group.[37] In another study, HMR 3004 administration in a model infected with viable *S. pneumoniae* also resulted in a decrease in pulmonary oedema formation. Lung tissue from treated animals resembled specimens obtained from uninfected controls.[32]

Thus, macrolides affect structural cells of the respiratory tract in case of acute inflammation. The emerging picture is that macrolides improve endothelium integrity, which results in less transendothelial migration of inflammatory cells. Furthermore, the reduced expression of adhesion molecules on both endothelium and epithelium may lead to less effective adhesion and diapedesis of PMNs and less accumulation of these cells in the lung compartment. This supposed reduction in PMN accumulation in lungs has already been confirmed in studies mentioned earlier. Moreover, murine studies showed

Table 5. The effect of macrolides on histological signs of inflammation in murine pneumonia models sorted by pathogen/stimulation.

Pathogen/stimulation	Macrolide	Histopathological findings	Comments	Reference
S. pneumoniae	HMR 3004	reduced lung oedema, lung tissue resembled healthy controls	compared with untreated controls	Duong *et al.* (2001)[32]
S. pneumoniae, macrolide resistant	RXM	less damage to lung tissue	compared with untreated controls	Yasuda *et al.* (2007)[35]
S. pneumoniae, killed	HMR 3004	reduced lung oedema, lung tissue resembled healthy controls	compared with untreated controls	Duong *et al.* (1998)[37]
M. pneumoniae	CLR	HPS significantly reduced	compared with placebo	Hardy *et al.* (2003)[31]
M. pneumoniae	CLR	HPS significantly reduced	compared with placebo	Tagliabue *et al.* (2008)[33]
M. pneumoniae	AZM	HPS significantly reduced	compared with placebo	Rios *et al.* (2005)[29]
M. pneumoniae	CET	HPS significantly reduced	no significant reduction of bacterial cultures in BALF	Rios *et al.* (2004)[30]
M. pneumoniae, killed	CLR	no reduction in HPS	compared with placebo	Hardy *et al.* (2003)[31]
M. pneumoniae antigen	CLR	CLR moderated the severity of the induced pneumonia	CIP and MIN did not decrease inflammation	Kurata *et al.* (2010)[20]
M. pneumoniae antigen, macrolide resistant	CLR	CLR did not moderate the severity of the induced pneumonia	CIP and MIN did not decrease inflammation	Kurata *et al.* (2010)[20]
H. influenzae, macrolide resistant	CLR	only mild inflammatory changes were evident	compared with untreated controls	Nakamura *et al.* (2010)[34]

AZM, azithromycin; BALF, bronchoalveolar lavage fluid; CLR, clarithromycin; CET, cethromycin; CIP, ciprofloxacin; HPS, histopathological score; MIN, minocycline; RXM, roxithromycin.

that macrolides reduced inflammatory signs caused by viable and non-viable bacteria, and even bacterial products. This not only suggests the existence of an immunomodulatory effect of macrolides in acute inflammation, but also points towards potential use as an immunomodulatory drug in clinical practice.

Conclusion

To our best knowledge, this is the first review to discuss the immunomodulatory effects of macrolides on the immune response in CAP. Overall, the existing evidence suggests that macrolides can temper the inflammatory response independent of antibacterial activity on different levels (cytokines, inflammatory cells, structural cells). The findings from the studies discussed in this review are in favour of the use of macrolides as adjuvant therapy in CAP.

However, the available data do not allow us to draw definite conclusions about individual macrolides, microorganisms or specific cytokines because of the heterogeneity of the studies. Moreover, *in vitro* and *in vivo* studies evaluating the immunomodulatory effects of macrolides in combination with β-lactam therapy are lacking. Such studies would be particularly clinically relevant, since worldwide controversy exists whether macrolides should be added to β-lactam antibiotics empirically in the treatment of CAP. To resolve this ongoing debate, at first the exact mechanisms of macrolide immunomodulation need to be elucidated, focussed on macrolides in combination with β-lactam antibiotics. Secondly, a randomised controlled trial is required to definitively assess the clinical impact of adjuvant macrolide therapy in CAP.

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Adjuvant macrolides attenuate the pro-inflammatory cytokine response to heat-killed *Streptococcus pneumoniae*

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Submitted

Abstract

Background

The current knowledge from *in vitro* and *in vivo* studies on the mechanisms of immunomodulation by macrolide antibiotics during acute infection is limited. Therefore, in this study, we investigated the effect of (adjuvant) macrolides on the cytokine response from human whole blood stimulated *in vitro* with *Streptococcus pneumoniae*.

Methods

Whole blood from healthy individuals was (pre)incubated during one hour with erythromycin, amoxicillin, or the combination of both antibiotics. Subsequently, whole blood was incubated for 24 hours with either heat-killed, viable or macrolide-resistant *S. pneumoniae*. The levels of interleukin-1β (IL-1β) IL-6, IL-8, IL-10, IL-12(p40), IL-17, IL-1 receptor antagonist (IL-1ra), monocyte chemotactic protein-1 (MCP-1), interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), macrophage inflammatory protein-1α (MIP-1α) were measured in the supernatants.

Results

Incubation of whole blood with a macrolide-containing antibiotic regimen before stimulation with heat-killed *S. pneumoniae* resulted in a trend towards lower levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and MCP-1, compared to β-lactam monotherapy. No difference in cytokine response was found when whole blood was stimulated with viable pneumococci (macrolide-sensitive or macrolide-resistant).

Conclusions

This study showed that macrolide therapy alone or combined with β-lactam antibiotics leads to a lower pro-inflammatory cytokine response in human whole blood stimulated with heat-killed *S. pneumoniae*, suggesting an immunomodulatory effect. This effect was not detected in case of stimulation with viable pneumococci, which as such caused a much higher cytokine response. More research, both experimental and clinical, is needed to further explore the immunomodulatory capacities of adjuvant macrolide therapy and the underlying mechanisms.

Introduction

Despite advances in antimicrobial therapy and vaccination strategies, community-acquired pneumonia (CAP) is still a highly prevalent disease that causes considerable morbidity and mortality.[1] Therefore, new additional treatment strategies are needed to improve the outcome of CAP. Modulation of the host immune response during CAP might be a potential strategy. The inflammatory host response is pivotal for defence against invading pathogens, but, when excessive, can cause local and systemic damage. Macrolide antibiotics are known to exert immunomodulatory activities next to their direct antibacterial effects.[2] Adjuvant macrolide therapy might therefore be a promising treatment option in CAP.

Empirical treatment of CAP generally contains a β-lactam antibiotic, targeted at *S. pneumoniae*, the most common causative microorganism in CAP.[3-5] β-lactam antibiotics are cell wall active agents that cause lysis of bacteria. This leads to the release of an array of bacterial components at the site of infection, further amplifying the pro-inflammatory immune response.[6, 7] To date, several observational and retrospective studies have suggested that the addition of a macrolide (protein synthesis inhibitors) to β-lactam antibiotics results in lower mortality among patients with CAP.[8-13] In addition to these clinical studies, the effect of combination therapy versus β-lactam monotherapy on disease outcome has recently been studied in a murine model of post-influenza pneumococcal pneumonia.[14] This study showed that the combination of antibiotics including a macrolide prevented neutrophil accumulation in the lungs and reduced mortality. A possible explanation for this beneficial effect of macrolides might be that macrolides can dampen the excessive immune activation caused by β-lactam therapy, and hereby improve outcome.[15]

Nevertheless, the clinical benefits of adjuvant macrolide therapy have not been firmly established yet, because not all studies have found a benefit of macrolides and well-designed randomised controlled trials are lacking.[16-20] Moreover, the current knowledge on the mechanisms of macrolide immunomodulation during acute inflammation from *in vitro* and *in vivo* studies is limited. Specifically, the influence of adjuvant macrolide administration on the cytokine response has not yet been investigated.

In this study, we investigated the effect of adjuvant macrolide antibiotics on the cytokine response in an *in vitro* model of acute infection with *S. pneumoniae*. We hypothesised that adjuvant macrolides will exert immunomodulatory activities beyond their antimicrobial effect during acute infection with *S. pneumoniae*, leading to a lower pro-inflammatory cytokine response. In a first series of experiments, we eliminated the direct antimicrobial effect of macrolides by stimulating whole blood with heat-killed *S. pneumoniae*. This way, if present, the immunomodulatory activity of macrolides could be studied in an isolated way. In a second series of experiments, whole blood was stimulated with viable *S. pneumoniae* in order to measure the cumulative effect of both macrolide immunomodulatory and antimicrobial activities. Finally, we evaluated whether an immunomodulatory effect is still present when the infecting microorganism would be macrolide-resistant.

Materials and methods

In vitro infection model

Peripheral blood was collected from healthy adult volunteers in a sodium heparin collection tube. Whole blood was diluted tenfold with RPMI Glutamax 1640 + 1% l-glutamine in 96-well round bottom plates (Nunc, Rochester, NY, USA). Subsequently, whole blood was incubated during one hour with erythromycin, amoxicillin, the combination of erythromycin with amoxicillin or no antibiotics at all. Afterwards, whole blood was incubated with or without a volume of heat-killed bacteria suspension corresponding to 107 microorganisms/mL in culture, and incubated for an additional 24 hours at 37°C, in an atmosphere of 5% CO2 and 100% relative humidity. Phytohaemagglutinin (PHA) in a concentration of 50 µg/mL was used as positive control. RPMI Glutamax 1640 + 1% l-glutamine was used as negative control. After incubation, cell-free culture supernatants were collected and stored at -70°C until analysis. Cell viability was determined by trypan blue (Sigma-Aldrich, St. Louis, MO, USA) exclusion after lysis of erythrocytes with hypertonic NaCl.

Infectious agents

S. pneumoniae serotype 19F and macrolide-resistant *S. pneumoniae* serotype 9 subtype V were cultured overnight on blood agar plates in an incubator at 37°C, 5% CO2. The bacteria were collected and suspended in M199 medium (Gibco by invitrogen Carlsbad, CA, USA) and cultured for an additional 4 hours. The number of microorganisms was determined using a Bürker-Türk counting chamber under a light microscope. Subsequently, the bacteria were centrifuged

at 3000 rpm (Hettich, Tuttlingen, Germany), washed with phosphate buffered saline (PBS; Gibco by invitrogen Carlsbad, CA, USA), inactivated by heating to 60°C for 60 minutes and resuspended in RPMI 1640 Glutamax™ (Gibco) to a concentration of 1.5x108 microorganisms/mL. The minimum inhibitory concentrations (MICs) were determined by microdilution techniques. For *S. pneumoniae* serotype 19 the MICs were <0.016 µg/mL for amoxicillin and 0.094 µg/mL for erythromycin. For macrolide-resistant *S. pneumoniae* serotype 9 V the MICs were <0.016 µg/mL for amoxicillin and >256 µg/mL for erythromycin.

Antibacterial agents

Erythromycin lactobionate (Erythromcin IV 1gr, Amdipharm, Basildon, Essex, England) was dissolved to a final concentration of 10 µg/mL in culture. Amoxicillin (Amoxicillin CF 500 mg, Centrafarm, Etten-Leur, The Netherlands) was dissolved to a concentration of 9 µg/mL in culture. The antibiotic concentrations used *in vitro* correspond to the maximum concentration obtained in blood during *in vivo* use.

Multiplex cytokine and chemokine assay

Concentrations of the cytokines interleukin-1β (IL-1β), IL-6, IL-10, IL-12(p40), IL-17, IL-1 receptor antagonist (IL-1ra), interferon-γ (IFN- γ) and tumour necrosis factor-α (TNF-α), and the chemokines monocyte chemotactic protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1α (MIP-1α/CCL3) and IL-8 (CXCL8), were measured by Milliplex multi-analyte profiling (HCYTOMAG-60K-11, Millipore, Billerica, MA, USA), essentially according to manufacturer's instructions. Data acquisition and analysis was performed on a Luminex 100 instrument (Luminex, Austin, TX, USA). The minimum detectable concentration for each cytokine was as follows: 0.8 pg/ml, IL-1β; 0.9 pg/ml, IL-6; 0.4 pg/ml, IL-8; 1.1 pg/ml, IL-10; 7.4 pg/ml, IL-12(p40); 0.7 pg/ml, IL-17; 8.3 pg/ml, IL-1ra; 1.9 pg/ml, MCP-1; 0.8 pg/ml, IFN-γ; 0.7 pg/ml, TNF-α; 2.9 pg/ml, MIP-1α.

Statistical analysis

Cytokine data are presented as median levels with interquartile ranges (IQR) because of a non-normal distribution. The statistical significance of differences was tested with either the Mann-Whitney U-test or the Kruskall-Wallis test, where appropriate. All statistical analyses were conducted using SPSS version 18.0 for Windows (Chicago, IL, USA). A two-sided p-value of <0.05 was considered to be statistically significant.

Table 1. Cytokine secretion from whole blood preincubated with various antibiotic regimens after 24 hours of incubation with

	Heat-killed *S. pneumoniae*				
	Medium only	Pathogen	Erythromycin	Amoxicillin	Combination
	n=7	n=15	n=15	n=15	n=14
IFN-γ	0,03 (0,00-0,03)*	0,6 (0,58-1,1)*	0,8 (0,28-1,4)	0,6 (0,28-1,1)	0,8 (0,39-1,5)
IL-10	0,96 (0,00-2,4)	2,0 (1,2-3,6)	1,5 (0,96-3,2)	2,0 (1,4-2,7)	2,6 (1,5-4,6)
IL-1ra	0 (0,00-0,00)*	26,8 (21,0-32,6)*	13,0 (4,7-21,0)	17,1 (6,9-24,9)	18,0 (10,4-27,3)
IL-12(p40)	0,00 (0,00-3,8)*	3,8 (2,3-5,4)*	4,5 (2,3-5,1)	2,3 (0,87-3,8)	2,3 (0,67-5,5)
IL-1β	0,00 (0,00-0,75)*	17,1 (10,3-20,3)*	9,8 (7,4-22,9)	13,5 (9,8-24,4)	11,1 (6,4-13,2)
IL-6	0,17 (0,00-0,35)*	27,8 (17,0-45,0)*	17,1 (12,7-23,4)†	26,4 (20,0-48,0)†	21,1 (13,8-29,0)
TNF-α	0,70 (0,52-0,79)*	32,4 (22,4-64,9)*	26,9 (19,9-60,2)	43,0 (32,0-59,1)	38,0 (26,8-47,0)
IL-8	24,2 (6,5-33,6)*	1147,2 (619,7-2061,3)*	920,9 (749,0-1198,5)†	2258,0 (998,8-2896,1)†	1082,7 (756,8-1803,4)
MCP-1	112,0 (29,9-254,5)*	6587,5 (4159,9-8011,4)*	5083,9 (4182,0-6612,2)	6795,7 (3571,0-8487,1)	3489,2 (2046,5-7602,5)
MIP-1α	0,00 (0,00-0,00)*	166,1 (127,7-202,5)*	133,0 (102,7-161,8)	207,0 (103,95-341,3)	128,4 (89,4-230,5)

Data are presented as median values (IQR) in pg/mL. * represents a significant difference between RPMI and pathogen a significant difference between pathogen and erythromycin (p-value <0.05). Abbreviations: Amoxi, amoxicillin; Combi, MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; TNF-α, tumour necrosis

Results

Cytokine secretion from whole blood after stimulation with heat-killed *S. pneumoniae*

Stimulation of whole blood with heat-killed *S. pneumoniae*, without antibiotic preincubation, significantly induced the secretion of cytokines and chemokines (Table 1). Preincubation with a macrolide-containing regimen (macrolides alone or combined with β-lactam antibiotics) resulted in an obvious trend towards lower levels of pro-inflammatory cytokines IL-1β and IL-6, anti-inflammatory cytokine IL-1ra, and chemokines IL-8, MCP-1 and MIP-1α. In particular, the concentrations of IL-6 and IL-8 decreased significantly under macrolide antibiotics, compared to β-lactam antibiotics (p=0.02 for both IL-6 and IL-8). Remarkably, preincubation with β-lactams alone resulted in higher levels of TNF-α, IL-8, MCP-1 and MIP-1α, compared to no antibiotic preincubation. Figure 1 summarises the results of four cytokines and chemokines that showed an obvious decrease under macrolide therapy.

heat-killed (left panel) or viable S. pneumoniae serotype 19F (right panel).

	Viable *S. pneumoniae*				
	Medium only	Pathogen	Erythromycin	Amoxicillin	Combination
	n=3	n=4	n=4	n=4	n=4
IFN-γ	0,62	178,1 (132,9-193,4)	77,2 (49,6-172,4)	62,4 (56,3-106,6)	36,6 (27,7-63,7)
IL-10	1,2	73,4 (67,0-88,3)‡	150,8 (140,4-162,1) ‡	129,4 (126,1-138,3)	164,8 (110,4-185,8)
IL-1ra	0	35,8 (24,7-38,0) ‡	47,1 (44,1-61,7) ‡	44,1 (40,3-47,9)	43,3 (34,3-52,4)
IL-12(p40)	0	133,5 (117,0-147,7)	89,5 (73,0-136,5)	87,2 (80,7-87,9)	67,7 (62,3-83,9)
IL-1β	6,6	6709,6 (6311,9-7172,2) ‡	1219,7 (999,9-1459,3) ‡	1123,9 (1028,8-1179,5)	1010,2 (714,9-1059,8)
IL-6	12	2153,4 (2033,7-2438,5) ‡	3042,6 (2681,4-3504,4) ‡	3199,6 (2558,4-3630,4)	3718,9 (2173,8-3959,1)
TNF-α	2,9	3760,4 (3071,5-3954,8) ‡	2001,6 (1728,6-2249,7) ‡	1626,0 (1461,8-1664,2)	1625,4 (1255,3-1939,7)
IL-8	74,2	10276,1 (9769,7-10519,1)	10410,0 (10279,5-10769,4)	11135,0 (10598,7-11525,3)	10693,9 (9904,7-11187,7)
MCP-1	322,9	4432,9 (4123,2-5432,7) ‡	11251,6 (8835,8-12019,5) ‡	10803,9 (9268,5-11589,1)	11574,3 (10539,3-11971,6)
MIP-1α	12	10784,3 (7533,2-12986,4)	7655,8 (4868,7-13575,0)	9802,6 (8289,8-12156,5)	7662,0 (6286,9-14621,2)

(p-value < 0.05). † represents a significant difference between erythromycin and amoxicillin (p-value < 0.05). ‡ represents erythromycin and amoxicillin; Ery, erythromycin; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; factor-α.

In summary, macrolide preincubation of whole blood attenuates the secretion of pro-inflammatory cytokines and chemokines after stimulation with heat-killed *S. pneumoniae*. β-lactam antibiotics, on the other hand, appeared to enhance the secretion of several cytokines and chemokines.

Cytokine secretion patterns in whole blood after stimulation with viable *S. pneumoniae*

Overall, viable bacteria elicited a much higher cytokine response than non-viable (heat-killed) bacteria (Table 1). When antibiotics were added to culture, the concentration of pro-inflammatory cytokines IL-1β, IL-12(p40) and TNF-α and chemokine MIP-1α decreased considerably. Nevertheless, the concentrations remained relatively high despite effective treatment that did not allow outgrowth of the microorganism. Comparison of the various antibiotic regimens tested showed no additional benefit of macrolide-containing regimens over β-lactams alone.

In summary, in experiments with viable pneumococci, no clear differences in cytokine secretion were found between the various antibiotic regimens.

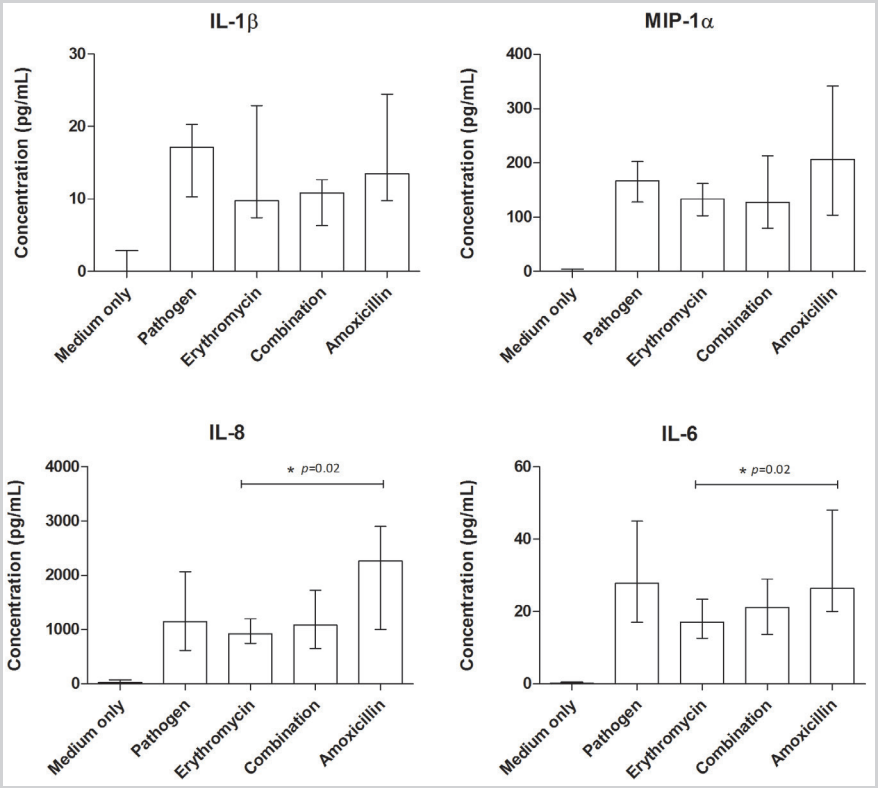

Figure 1. The secretion of IL-1β, MIP-1α, IL-8 and IL-6 from whole blood stimulated with heat-killed *S. pneumoniae* serotype 19F and treated with various antibiotic regimens after 24 hours of incubation. Data shown are the median (with interquartile ranges) of 30 or 29 independent experiments. In PHA stimulated cultures, 1.20 (IQR 0.11-27.24) pg/ml IL-1β, 0.00 (IQR 0.00-1841.17) pg/ml MIP-1α, 35.26 (IQR 29.98-7763.41) pg/ml IL-8, and 0.35 (IQR 0.00-687.69) pg/ml IL-6 was produced.

Cytokine secretion from whole blood after stimulation with macrolide-resistant *S. pneumoniae*

The results of these experiments are summarised in Table 2. As expected, the addition of β-lactams to whole blood stimulated with macrolide-resistant microorganisms resulted in decreased concentrations for most cytokines. This effect is thought to be due to the direct antibacterial effect of the β-lactam antibiotic. In contrast, incubation with macrolide antibiotics did not lead to a reduction of cytokine concentrations. When macrolides were added adjuvant to β-lactam antibiotics, no further reduction of cytokine levels was found, compared to β-lactams alone. Surprisingly, the cytokines IL-6 and IL-10 and the chemokines IL-8, MCP-1 and MIP1-α showed no decrease at all after treatment with any antibiotic regimen; their levels even increased.

Table 2. Cytokine secretion from whole blood stimulated with viable macrolide-resistant *S. pneumoniae* serotype 9V treated with various antibiotic regimens after 24 hours of incubation.

	Medium only n=3	Pathogen n=4	Erythromycin n=4	Amoxicillin n=4	Combination n=4
IFN-γ	0,62	119,9 (112,7-161,6)	109,9 (56,8-193,5)*	22,3 (7,1-44,9)*	19,1 (6,8-49,6)
IL-10	1,2	22,0 (15,2-39,4)†	135,0 (109,0-212,1)†	120,1 (91,3-206,3)	118,0 (106,3-129,7)
IL-1ra	0,00	95,2 (87,0-104,7)	80,3 (73,7-115,6)*	56,3 (49,8-65,1)*	44,8 (39,5-57,0)
IL-12(p40)	0,00	75,7 (53,5-95,0)	112,6 (75,7-132,9) ‡	39,6 (33,0-76,6)	47,3 (34,6-56,0)‡
IL-1β	6,6	7242,5 (6674,1-7587,5)	6910,0 (6261,2-8240,4)* ‡	713,9 (393,1-1241,4)*	727,8 (658,1-776,4) ‡
IL-6	12,0	1266,7 (1191,1-1643,5) †	2297,5 (2058,2-4736,0) †	3117,0 (2479,2-4325,6)	2691,3 (2445,4-2720,1)
TNF-α	2,9	2374,1 (2239,9-2871,8) †	3377,8 (3080,5-4246,4) *†‡	970,8 (672,2-1644,2)*	1050,7 (892,2-1211,0) ‡
IL-8	74,2	8891,9 (8601,5-9804,3)	10283,6 (9940,8-10706,2)	10286,5 (1007,8-10475,4)	10287,6 (10069,8-10553,1)
MCP-1	322,9	3141,0 (2756,7-3887,0) †	7523,6 (6122,9-9941,5) †	10907,8 (9005,7-11900,8)	9690,1 (8715,3-10981,6)
MIP-1α	12,0	4700,6 (4005,0-5456,5)	5824,0 (5402,2-10614,1)	7172,3 (6594,8-9009,7)	6979,0 (5422,8-7474,9)

Data are represented as median (IQR) in pg/mL. * a significant association with a p-value <0.05.
Abbreviations: Amoxi, amoxicillin; Combi, erythromycin and amoxicillin; Ery, erythromycin; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; TNF-α, tumour necrosis factor-α.

In summary, in these experiment with macrolide-resistant pneumococci, (adjuvant) macrolides had no downregulatory effect on the cytokine response from whole blood.

Discussion

Our study has two main findings. First, in whole blood stimulated with heat-killed *S. pneumoniae*, macrolide administration (alone or in combination with β-lactam antibiotics) resulted in lower levels of several pro-inflammatory cytokines and chemokines, when compared with only β-lactams. This suggests an immunomodulatory effect of macrolide antibiotics. Second, stimulation of whole blood with viable *S. pneumoniae* induced an overwhelming cytokine response. In these experiments, no difference in cytokine response was detected between a macrolide-containing antibiotic regimen and β-lactams alone.

The present experiments with heat-killed pneumococci demonstrate that macrolides are able to attenuate the production of pro-inflammatory cytokines, independent of their direct antimicrobial effect. Previous studies using human *in vitro* models or murine pneumonia models have also reported an immunomodulatory effect of macrolides.[21] However, the information from former studies is limited by the fact that in most cases only macrolides have been used. Up to now, only one study in mice with pneumococcal pneumonia following influenza virus infection has investigated the effect of macrolide and β-lactam combination therapy on outcome. Combination therapy resulted in a better outcome, but how this clinical effect correlates with cytokine production patterns was not investigated.[14] We are the first to demonstrate that macrolides might dampen of the cytokine response when administered adjuvant to β-lactam antibiotics. When we would extrapolate these findings to clinical practice, this may mean that adjuvant macrolide therapy can modulate inflammation in patients with CAP and thereby prevent immunopathology following β-lactam therapy. In previous studies, lower levels of pro-inflammatory cytokines have been correlated with favourable outcome in CAP.[22] This supports combination therapy over β-lactam monotherapy in the treatment of CAP.

In the experiments with viable pneumococci (either macrolide-sensitive or macrolide-resistant), however, no indications for immunomodulation by macrolides were found. This may indicate a true absence of immunomodulatory activities. It can also be that the immunomodulatory effect was obscured

because of the overwhelming cytokine response. This might be due to the high inoculum size of the microorganisms, which is the upper range of the bacterial load that has been found *in vivo* in CAP, with or without sepsis.[23] Alternatively, it is possible that the effect of the antibiotics on the bacteria did not yet manifest in terms of lower cytokine secretion. In the experiments with macrolide-resistant pneumococci (sensitive to β-lactams), as expected, β-lactam antibiotics were the most effective in tempering the inflammatory response, probably by decreasing the bacterial load.

It must be emphasised that the current experiments in human whole blood are only an approximation of the actual immune response in patients with CAP. Before translation of the results to clinical practice, some important aspects of macrolide immunomodulation need further elucidation. First, it is not known whether lowering of the pro-inflammatory cytokine response can have a negative effect on the clearance of (perhaps inactivated or lysed) pneumococci in the lung. Second, the ratio of cytokine levels in serum relative to the levels in the lung(s) during acute infection is unknown and it is unclear how this ratio is influenced by antibiotic therapy. Furthermore, a potential pitfall of immunomodulatory or immunosuppressive therapy during acute infection is the so-called immune paralysis or compensatory anti-inflammatory response syndrome.[24] During infection, a blend of pro-inflammatory, anti-inflammatory and other immunoregulatory molecules circulate in the blood. In the early stages of infection, the net effect of these mediators is a pro-inflammatory response that primes the host for clearing the infection. Afterwards, however, the pro-inflammatory state can dwindle and the host may shoot into an anti-inflammatory state so severe that no inflammatory response can be mounted, even after a new infectious insult. Hence it appears that inhibition of an excessive initial pro-inflammatory response by immunomodulatory or immunosuppressive agents can be beneficial, but an intervention that prolongs or even aggravates the period of immune paralysis is undesirable. Thus, to ultimately yield the best results from any immunomodulatory or immunosuppressive therapy during acute infection, future studies should map this biphasic response and determine the optimal timing of administration of immunomodulating agents.

There are other limitations, inherent to *in vitro* experimentation, that affect the utility of the presented findings. First, the cytokine response in an *in vitro* study takes place in a closed system that does not allow diffusion of cytokines or

movement of cells. This is in contrast with *in vivo* systems where free movement of humoral and cellular components is possible, and therefore our experiments gave no reliable representation of the natural response. Fortunately, it is not necessary to mirror the exact natural response to determine whether cytokine secretion is affected by macrolides. Second, although no diffusion takes place, the cytokine concentrations are subject to a limited half-life time that affects the detected levels.[25] Third, the maximum incubation time of whole blood is limited by the lifespan of granulocytes. The maximum incubation time of 24 hours may not suffice to induce the production of all cytokines.[26] Furthermore, in all experiments, IL-17 was found to remain below the detection limit (0.7 pg/ml) and therefore was not reported in our results. Finally, cytokines are not the only mediators in an inflammatory response, and identification and monitoring of molecules that affect adhesion and the coagulation cascade during infection could also complement our knowledge on the mechanisms of immunomodulation by macrolides.

In conclusion, our data show that the administration of macrolide antibiotics alone or in combination with β-lactam antibiotics to human whole blood stimulated with heat-killed *S. pneumoniae* leads to a lower pro-inflammatory cytokine response, compared to β-lactam alone, suggesting an immunomodulatory effect. This effect was not detected in case of stimulation with viable *S. pneumoniae*. More experiments are needed to further explore the immunomodulatory capacities of adjuvant macrolide therapy and the underlying mechanisms. Moreover, to conclusively assess whether macrolide combination therapy is indeed superior in the treatment of CAP, a benefit compared to β-lactam monotherapy needs to be confirmed in clinical studies.

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IV

Immunomodulation by vitamin D

Addition of vitamin D status to prognostic scores improves the prediction of outcome in community-acquired pneumonia

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Abstract

Background

Vitamin D plays a role in host defence against infection. Vitamin D deficiency is common worldwide. The prognostic value of vitamin D levels in pneumonia is unknown. In this study, we aimed to investigate the impact of vitamin D status on outcome in community-acquired pneumonia (CAP).

Methods

We conducted a prospective cohort study in 272 hospitalised patients with CAP. Levels of 25-hydroxyvitamin D, leukocytes, C-reactive protein and total cortisol, and the Pneumonia Severity Index (PSI) and CURB-65 scores were measured on admission. Major outcome measures were intensive care unit (ICU) admission and 30-day mortality.

Results

143 patients (53%) were vitamin D deficient (<50 nmol/L), 79 patients (29%) were vitamin D insufficient (50-75 nmol/L) and 50 patients (18%) were vitamin D sufficient (>75 nmol/L). Vitamin D deficiency was associated with an increased risk of ICU admission and 30-day mortality. Vitamin D status was an independent predictor of 30-day mortality (area under the curve (AUC) = 0.69; 95% CI 0.57-0.80). Multivariate regression analysis including all predictors for outcome resulted in a final model including vitamin D status and the PSI score, with a significantly higher prognostic accuracy compared with the PSI score alone (AUC = 0.83; 95% CI 0.71-0.94).

Conclusions

Vitamin D deficiency is associated with adverse outcome in CAP. Vitamin D status is an independent predictor of 30-day mortality and adds prognostic value to other biomarkers and prognostic scores, in particular the PSI score.

Introduction

Community-acquired pneumonia (CAP) is a common disease with considerable morbidity and mortality, despite preventive vaccinations and effective antibiotic treatment. Together with influenza, CAP is the eighth leading cause of death in persons aged >65 years in the United States and is the leading infectious cause of death worldwide.[1] New targets are needed in the assessment and management of CAP, to better guide the therapeutic options and ultimately improve clinical outcome.

Recently, there has been much interest in the role of vitamin D in host defence against infection. Apart from its classical function in calcium-phosphate homeostasis, vitamin D has pleiotropic immunomodulatory properties. Vitamin D plays an important role in the innate immune response, in particular by increasing the production of antimicrobial peptides (β-defensin, cathelicidin).[2] Furthermore, vitamin D has profound effects on the activity of the adaptive immune system through its interaction with the vitamin D receptor expressed by dendritic cells, monocytes, T-cells and B-cells.[2]

Vitamin D deficiency is very common worldwide, particularly in risk groups such as the elderly and people living distant from the equator.[3, 4] The main sources of vitamin D in humans are synthesis in the skin following exposure to ultraviolet B radiation in sunlight, diet, and dietary supplements. Accordingly, vitamin D deficiency can result from inadequate sun exposure, insufficient nutrition, or malabsorption.

Several studies have shown an association between vitamin D deficiency and increased susceptibility to respiratory tract infections.[5-9] Up to now, only one study has investigated the relationship between 25-hydroxyvitamin D levels and clinical outcome in adult patients with CAP.[10] Vitamin D deficiency (serum 25-hydroxyvitamin D <50 nmol/L) was present in 44% of the patients, of which 15% were severely deficient (serum 25-hydroxyvitamin D <30 nmol/L). In this study, vitamin D deficiency was associated with increased 30-day mortality in patients admitted to the hospital with CAP during wintertime. However, this study lacked information about the prognostic value of vitamin D status.

Because the prognostic value of vitamin D status in CAP is currently unknown, we undertook this study to determine the impact of vitamin D status on clinical outcome in a well-defined cohort of CAP patients in the Netherlands, a Western European country which is located on the latitude of 52° North. We investigated the contribution of vitamin D status to the prognostic accuracy of other biomarkers and commonly used prognostic scores. Our hypothesis is that

a low 25-hydroxyvitamin D level is related to adverse outcome, due to the lack of immunomodulatory activities in case of deficiency.

Methods

Patients and study design

This is a prospective cohort study that uses data from adult patients who participated in a randomised clinical trial that examined the effectiveness of dexamethasone in CAP. The details of the study population have been described previously and are summarised in Appendix A.[11] The pneumonia severity index (PSI) score and CURB-65 score were calculated on admission.[12, 13] Drug use before hospital admission (prescribed by the general practitioner) was registered. Patients receiving calcitriol or 1-α-hydroxyvitamin D3 were excluded, because these are likely to influence vitamin D status but are not measured by the 25-hydroxyvitamin D assay used. Patients receiving other formulations of vitamin D supplementation were included. The study was approved by the local Medical Ethics Committee and all patients gave written informed consent.

Outcome measures

The primary endpoint was adverse clinical outcome, defined as the need for intensive care unit (ICU) admission during hospitalisation or death within 30 days of hospital admission (30-day mortality). Patients who were admitted for the ICU were in need of mechanical ventilation, vasopressor support or both.

Serum vitamin D measurement

Blood serum samples were collected on admission and stored at -80^{0}C. Serum 25-hydroxyvitamin D level was measured with the commercially available 25(OH)D TOTAL Liaison® chemiluminescence assay (Liaison, Diasorin S.p.A., Saluggia, Italy). Based on this level, patients were categorised as deficient (<50 nmol/L), insufficient (50-75 nmol/L) and sufficient (>75 nmol/L).[14]

Measurement of other biomarkers

Leukocytes, C-reactive protein (CRP) and total cortisol were measured in serum on the day of presentation. Concentrations of CRP were measured with high-sensitivity CRP (Roche Diagnostics GmbH, Mannheim, Germany). The total serum cortisol level was measured with a solid-phase competitive ELISA (Calbiotech, Spring Valley, USA).

Statistical analysis

Baseline characteristics were compared among the categories of vitamin D status using the Chi-square test, the Fisher's Exact-test, the one-way analysis of variance or the Kruskal-Wallis test, where appropriate. The association between vitamin D status and ICU admission or 30-day mortality was evaluated by univariate analysis, wherein vitamin D status was analysed as a categorical variable. To explore causality of the association, regression analysis was conducted focused on identifying confounding factors. To enlarge the number of confounders that could be tested, the combined endpoint mortality/ICU admission was used. We considered factors (from univariate analysis or previously mentioned in literature) as potential confounders when they were associated with both vitamin D deficiency and adverse outcome. We selected confounders for the multivariate model stepwise by direct estimation of the degree of confounding produced by each factor (relative change in the regression coefficient for adverse outcome associated with vitamin D deficiency). Factors that modified the regression coefficient by ≥10% were considered confounders. A final multivariate model was obtained including all identified confounding variables.

To examine the predictive value of vitamin D relative to other predictors for mortality from CAP, univariate and multivariate regression analysis was performed. All predictors significantly associated ($p<0.05$) with mortality in the univariate analysis were entered simultaneously into a multiple backward logistic regression model. Predictors were sequentially deleted from the initial model on the basis of lack of significance ($p<0.05$). To assess the discriminative qualities of the final model, Receiver Operator Characteristics (ROC) curve analysis was performed. The goodness of fit of the final model was tested with the Hosmer-Lemeshow test.

All statistical analyses were performed using SPSS 18.0 (Chicago, USA). A two-tailed p-value <0.05 was considered statistically significant.

Results

A total of 304 patients were included in this study. After the exclusion of 7 patients who were using 1-α hydroxyvitamin D3 and 25 patients missing a 25-hydroxyvitamin D value, 272 patients remained for analysis. Mean age was 63.5 years (SD 18.3) and 56% were male. In total, 15 patients (5.5%) were admitted to the ICU during hospital stay, of whom 4 patients died. At day 30, 256 patients (94%) had survived and 16 patients (5.9%) had died. Table 1 depicts the baseline characteristics of the study population, including stratification for vitamin D status.

Table 1. Baseline characteristics of 272 patients with community-acquired pneumonia, including stratification for vitamin D status

Characteristics	All patients n=272	Vit D <50 nmol/L n=143	Vit D 50-75 nmol/L n=79	Vit D >75 nmol/L n=50	p-value[a]
Sex, male	153 (56)	80 (56)	42 (53)	31 (62)	0.61
Age (years)	63.5 (18.3)	66.7 (18.6)	61.1 (17.7)	58.3 (16.8)	<0.01[c]
Race[b]					0.69
White	270 (99)	141 (99)	79 (100)	50 (100)	
Other	2 (0.7)	2 (1)	0 (0)	0 (0)	
Nursing-home resident	13 (5)	11 (8)	2 (3)	0 (0)	0.05[c]
Vitamin D supplementation	11 (4)	0 (0)	6 (8)	5 (10)	<0.01[c]
Comorbidities					
Liver disease	2 (0.7)	2 (1)	0 (0)	0 (0)	0.69
Renal disease	22 (8)	18 (13)	3 (4)	1 (2)	0.02[c]
Heart failure	45 (17)	34 (24)	9 (11)	2 (4)	<0.01[c]
Malignancy	17 (6)	11 (8)	3 (4)	3 (6)	0.63
COPD	29 (11)	22 (15)	3 (4)	4 (8)	0.02[c]
DM	39 (14)	28 (20)	8 (10)	3 (6)	0.03[c]
Laboratory parameters					
Albumin (g/L)	42.0 (8.0)	41.1 (8.4)	42.7 (6.8)	43.5 (8.6)	0.13
Leukocyte count (x10^9/L)	14.3 (6.5)	14.9 (7.0)	13.8 (6.5)	13.5 (4.8)	0.32
CRP (mg/L)	212.9 (139.5)	203.9 (143.6)	232.3 (125.2)	207.9 (148.6)	0.34
Cortisol (ng/ml)	221.9 (144.4-386.6)	255.2 (160.3-435.6)	196.4 (135.3-360.1)	186.0 (125.7-330.3)	0.02[c]
CURB-65 score	1.66 (1.2)	1.9 (1.2)	1.5 (1.2)	1.2 (1.1)	<0.01[c]
PSI score	90.1 (36.4)	98.1 (36.7)	83.3 (32.7)	78.1 (36.1)	<0.01[c]
PSI risk class					<0.01[c]
Class I-III	147 (54)	63 (44)	49 (62)	35 (70)	
Class IV-V	125 (46)	80 (56)	30 (38)	15 (30)	

Data are presented as number (%), mean (SD) or median (IQR).
[a] Comparison of the three 25-hydroxyvitamin D categories. [b] Self-reported. [c] Characteristics showing a significant association with a p-value <0.05.
Abbreviations: COPD, chronic obstructive lung disease; CRP, C-reactive protein; DM, diabetes mellitus; PSI, pneumonia severity index; Vit D, 25-hydroxyvitamin D.

Prevalence of vitamin D deficiency

In this cohort of Dutch patients with CAP, the median 25-hydroxyvitamin D level was 47.4 nmol/L (IQR 30.4-68.2). Overall, 143 patients (53%) were vitamin D deficient (<50 nmol/L), 79 patients (29%) were vitamin D insufficient (50-75 nmol/L) and 50 patients (18%) were vitamin D sufficient (>75 nmol/L). Median 25-hydroxyvitamin D levels were lowest in patients presenting during the winter (December 21st - March 20th; n=89, 36.2 nmol/L, IQR 25.9-51.4) and highest in patients presenting in the summer (June 21st- September 20th; n=35, 70.6 nmol/L, IQR 35.2-81.8). The prevalence of vitamin D deficiency per season is shown in Figure 1. The 11 patients receiving vitamin D supplements (other than 1-α hydroxyvitamin D3) had significantly higher 25-hydroxyvitamin D levels than patients without vitamin D supplementation (74.6 nmol/L, IQR 63.5-107.0, vs. 45.3 nmol/L, IQR 29.8-66.6) (p<0.01).

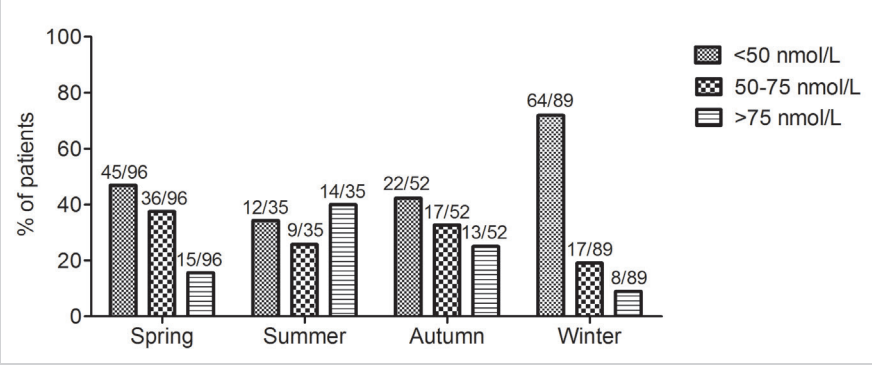

Figure 1. The prevalence of vitamin D deficiency throughout the astronomic seasons. The numbers above the bar indicate the number of patients within the related vitamin D category.

Association between vitamin D status and clinical outcome

Median 25-hydroxyvitamin D levels were significantly lower in patients who were admitted to the ICU, compared to patients without ICU admission (34.9 nmol/L, IQR 23.8-46.3 vs. 48.3 nmol/L, IQR 30.8-68.4) (p:0.04). Vitamin D deficiency was associated with a higher rate of ICU admission, compared with patients with vitamin D (in)sufficiency (Figure 2A).

Patients who died within 30 days had significantly lower 25-hydroxyvitamin D levels, compared with patients who survived (25.8 nmol/L, IQR 19.8-40.1 vs. 48.8 nmol/L, IQR 32.4-68.9) (p<0.01). Vitamin D deficiency was associated with a higher mortality rate, compared with patients with vitamin D (in)sufficiency (Figure 2B).

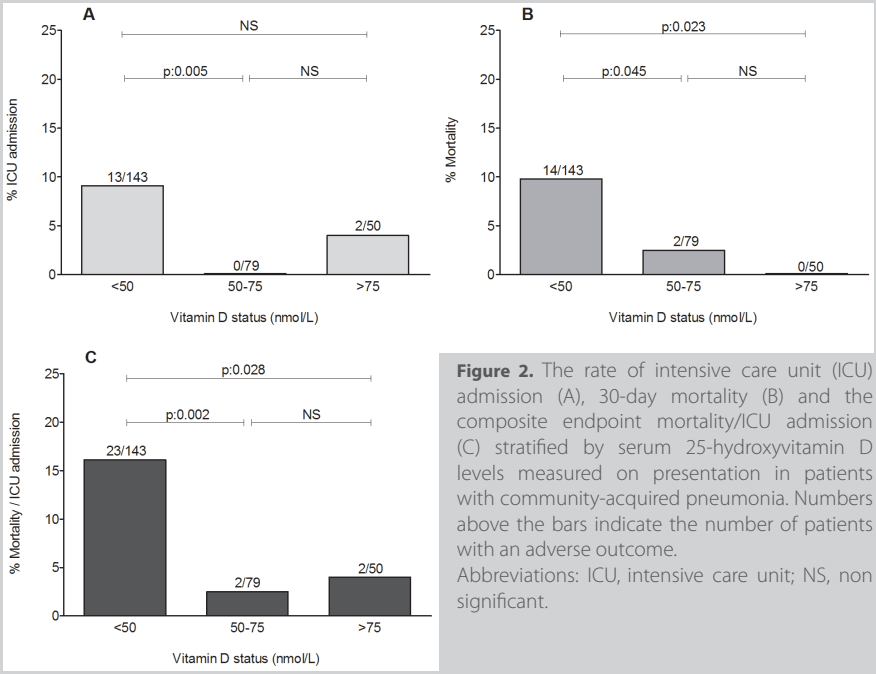

Figure 2. The rate of intensive care unit (ICU) admission (A), 30-day mortality (B) and the composite endpoint mortality/ICU admission (C) stratified by serum 25-hydroxyvitamin D levels measured on presentation in patients with community-acquired pneumonia. Numbers above the bars indicate the number of patients with an adverse outcome.
Abbreviations: ICU, intensive care unit; NS, non significant.

Identification of confounding

In the crude analysis of the association between vitamin D status and clinical outcome, vitamin D deficiency was associated with an OR of 4.60 (95% CI 1.04-20.27) for the composite endpoint mortality/ICU admission. To explore possible causality of this association, we aimed to identify confounding factors. The factors race, sex, age, season of admittance, nursing-home residency, liver disease, renal disease, heart failure, malignancy, COPD, diabetes mellitus and serum albumin concentration on admission were all considered as potential confounders. Appendix B lists the degree of statistical confounding by each factor in multivariate logistic regression analysis. Age and heart failure fulfilled our criteria of being confounders and were retained in the final multivariate regression model. Table 2 lists the unadjusted and adjusted ORs for the association between vitamin D status and clinical outcome. An obvious trend towards a higher risk of adverse outcome among patients with vitamin D deficiency was observed after adjustment for confounders.

Table 2. Odds Ratio's for the association between vitamin D status and clinical outcome: before and after adjusting for confounders

Vitamin D status	No. of patients (%) (n=272)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)[b]
25-hydroxyvitamin D >75 nmol/L	50 (18)	Reference	Reference
25-hydroxyvitamin D 50-75 nmol/L[a]	79 (29)	0.623 (0.085-4.573)	0.510 (0.068-3.807)
25-hydroxyvitamin D <50 nmol/L[a]	143 (53)	4.600 (1.044-20.272)	2.949 (0.640-13.598)

Clinical outcome was the composite endpoint mortality/intensive care unit admission.
[a] Compares patients with 25-hydroxyvitamin D levels between 50-75 nmol/L or <50 nmol/L with patients with 25-hydroxyvitamin D levels >75 nmol/L (reference category). [b] Adjusted ORs for the confounders age and heart failure. Abbreviations: CI, confidence interval; OR, odds ratio.

Vitamin D status as predictor for 30-day mortality from CAP

Based on the abovementioned association between vitamin D deficiency and adverse outcome, vitamin D status on the day of admission could be a useful prognostic biomarker in patients with CAP. To assess the potential to predict 30-day mortality, we compared the predictive value of vitamin D status with other commonly used predictors for prognosis in CAP by univariate and multivariate regression analysis. For statistical reasons (zero deaths within the category vitamin D sufficiency) and for practical application, we analysed vitamin D deficient patients (<50 nmol/L) versus other patients (≥50 nmol/L). We included the following predictors (all measured on the day of admission) in univariate regression analysis: vitamin D status (in 2 categories), leukocyte count (on a continuous scale), CRP level (on a continuous scale), cortisol level (on a continuous scale), PSI score (in 3 categories: 0-90 points, low risk; 91-130 points, intermediate risk; >130 points, high risk of 30-day mortality) and CURB-65 score (0-5 points, on a continuous scale). Four parameters appeared to be significant predictors for mortality, namely vitamin D status, cortisol level, PSI score and CURB-65 score. When vitamin D status was added to cortisol level, PSI score and CURB-65 score individually, all prognostic accuracies improved (see Appendix C). Multivariate regression analysis including all four parameters resulted in a superior final model containing vitamin D status and PSI score. The Hosmer-Lemeshow goodness of fit test for the final model was not significant (p:0.94), indicating that this model fits the data well. Figure 3 shows the ROC curves with the corresponding AUC for the final model, together with the ROC curves and AUCs of vitamin D status and PSI score separately. With an AUC of 0.83 (95% CI 0.71-0.94), the prognostic accuracy of the combination of vitamin D status and PSI score was superior to the accuracy of the other predictors or the PSI score alone (AUC = 0.78, 95% CI 0.64-0.91).

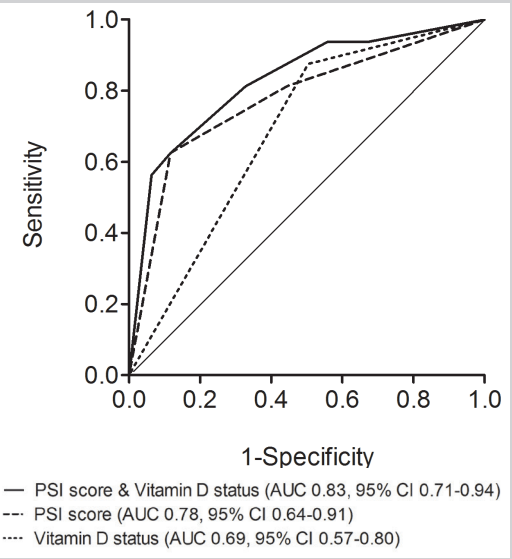

Figure 3. Receiver Operator Characteristics curve analysis of the prediction of 30-day mortality by Pneumonia Severity Inex score, vitamin D status, and the combined model 'PSI score & vitamin D status'. Data on the day of admission are shown.
Abbreviations: AUC, area under the curve; CI, confidence interval; PSI, Pneumonia Severity Index.

Discussion

Our study has three main findings. First, vitamin D deficiency is highly prevalent in this Dutch cohort of patients admitted to the hospital with CAP. Second, vitamin D deficiency is associated with adverse outcome. Third, vitamin D status on presentation is a significant predictor of 30-day mortality and adds predictive value to other biomarkers and clinical scores. The combination of PSI score and vitamin D status appeared to be the best predictive model for 30-day mortality in CAP.

Vitamin D deficiency is very common in many populations worldwide, especially during the winter season.[4, 15-19] Vitamin D deficiency is not limited to particular risk groups, such as housebound and institutionalized elderly: high rates have also been reported in normal urban populations.[15, 20] The high prevalence of vitamin D deficiency found in our cohort of CAP patients is comparable to that reported in former studies in the general elderly population in the Netherlands.[19, 21, 22]

To the best of our knowledge, only two studies in adults have investigated the association between vitamin D status and the course of infectious disease. Leow *et al*. explored the relationship between vitamin D and clinical outcome in 112 adults admitted with CAP.[10] This study was carried out during the winter in Hamilton, New Zealand, which is situated at a latitude of 37° South.

They observed that vitamin D deficiency is associated with increased 30-day mortality. Ginde *et al.* studied 81 adults who were evaluated for suspected infection at the emergency department.[23] They observed that vitamin D insufficiency is associated with higher sepsis severity. Our study confirms these previous findings (Figure 2).

In clinical practice, the PSI score and CURB-65 score are mostly used to predict 30-day mortality in patients with CAP. To date, several studies have investigated whether the inclusion of additional biomarkers would improve the 30-day mortality prediction of these two scores. Among others, the prognostic performance of CRP, procalcitonin, precursor peptides of endothelin-1, natriuretric peptides, pro-adrenomedullin and cortisol have been evaluated, with variable, and sometimes promising, results.[24-30] The prognostic value of serum 25-hydroxyvitamin D has never been investigated. The comparison of the prognostic value of vitamin D status on presentation to the predictive ability of other biomarkers and clinical scores on CAP outcomes is a novel aspect of this work. Our study suggests that vitamin D status adds prognostic value to other biomarkers and prognostic scores. The combination of PSI score and vitamin D status appeared to be superior in prediction of 30-day mortality in CAP, with an AUC of 0.83 (Figure 3). C-reactive protein and cortisol were both inferior to vitamin D status in our study. A possible explanation for the predictive value of vitamin D status in CAP might be an indirect effect of poor physical or nutritional status. A direct effect could be the proven immunomodulatory activity of vitamin D. Although our predictive model is promising, further substantiation in future studies is needed. Before introduction in clinical practice can be considered, a large prospective study is needed to ultimately determine the best combination of biomarkers and clinical scores for prediction of outcome in CAP.

The finding of an association between vitamin D deficiency and adverse outcomes raises the question whether vitamin D supplementation in the acute care of CAP will improve outcome. The high prevalence of vitamin D deficiency in patients with CAP makes vitamin D a potential candidate for adjuvant treatment strategies. Recently, two randomised controlled trials assessed the effects of vitamin D supplementation in young children admitted with pneumonia. Choudhary *et al.* demonstrated that short term supplementation with oral vitamin D (1,000-2,000 IU per day for 5 days) had no beneficial effect on the duration of resolution of severe pneumonia in children <5 years. Unfortunately, no 25-hydroxyvitamin D levels were measured in this study. Hence, it cannot

be ruled out that the recruited children were already vitamin D deficient and that the given doses were too low to cause an effect.[31] In the study of Manaseki *et al.*, a single high dose oral vitamin D3 (100,000 IU) upon admission did not result in a reduction of the duration of illness in children with pneumonia. However, they demonstrated a significant reduction in the occurrence of new episodes of pneumonia over a 90-day period.[32] The latter finding might also extend to adults, where an alternative option might be preventive vitamin D supplementation based on 25-hydroxyvitamin D measurement in high risk groups during wintertime, aimed at reaching a 25-hydroxyvitamin D level >75 nmol/L.[33, 34] Future research should further explore the capacity of vitamin D to play a preventive and/or therapeutic role in pneumonia.

Some limitations of our study should be mentioned. First, only a relatively small number of patients reached the study endpoint, which resulted in larger confidence intervals. This could have precluded reaching significance in some of the analyses, despite clear trends. Second, to date, there is still no consensus on optimal 25-hydroxyvitamin D levels as measured in serum. In our analyses, patients were classified into the categories deficient (<50 nmol/L), insufficient (50-75 nmol/L) and sufficient (>75 nmol/L).[14] However, in literature, different classifications of vitamin D status have been proposed. In order to rule out the possibility that other cut-offs would result in different predictive values, we conducted a sensitivity analysis by applying two other classifications (Appendix D).[3, 10] In both cases, the rate of adverse outcome remained significantly higher for vitamin D deficient patients. Interestingly, we observed that within the group of vitamin D deficient patients, a 25-hydroxyvitamin D level <25 nmol/L or <30 nmol/L was associated with an even higher rate of adverse outcome (OR 7.77, 95% CI 1.62-37.30 respectively OR 8.58, 95% CI 2.70-27.31). Thus, the lower range of 25-hydroxyvitamin D seems to provide more contrast for a predictive model and these cut-off values should therefore be further explored in future studies.

Finally, owing to the observational design of the study, we were not able to establish a causal relationship between vitamin D deficiency and adverse outcome. Although a trend towards higher risk persisted after adjustment for confounders, the presence of unmeasured confounding cannot be ruled out. Mainly, we lacked extensive information about nutritional status, Body Mass Index (BMI) and other lifestyle factors; nevertheless, we were able to include albumin as proxy for malnutrition.

In conclusion, vitamin D deficiency is highly prevalent in patients hospitalised with CAP in the Netherlands. We confirmed that vitamin D deficiency is associated with adverse outcome in CAP. Vitamin D status on presentation is a significant predictor for 30-day mortality, with additional prognostic value when combined with other biomarkers or prognostic scores, in particular the PSI score. Some clues for a possible causal relationship were found. Future studies should further explore the likelihood of a causal relation between vitamin D deficiency and adverse outcome. In the case of a causal relationship, vitamin D supplementation might be a promising candidate for adjuvant treatment in CAP. Meanwhile, vitamin D status on presentation can be used as a prognostic marker.

Appendix A

Details of the study population

Adult patients with confirmed CAP who presented at the emergency department of the St. Antonius Hospital in Nieuwegein or Gelderse Vallei Hospital in Ede in the Netherlands (both teaching hospitals) were enrolled between November 2007 and September 2010. The diagnosis of pneumonia was confirmed when a new pulmonary infiltrate on a chest radiograph was present in combination with at least two of the following criteria: cough, sputum production, temperature above 38^0C or below 35^0C, auscultatory findings consistent with pneumonia, C-reactive protein (CRP) concentration of more than 15 mg/L, white blood cell count of more than $10x10^9$ cells/L or lower than $4x10^9$ cells/L, or >10% of rods in leukocyte differentiation. Patients who were immunocompromised, on immunosuppressive therapy, or who required immediate admission to the intensive care unit (ICU) were excluded.

Appendix B

Identification of confounders in the association between vitamin D deficiency (25-hydroxyvitamin D <50 nmol/L versus >75 nmol/L (reference category)) and adverse outcome (composite endpoint mortality/ICU admission)

Potential confounders	Regression coefficient	Δ Regression coefficient (%)[a]	Odds ratio (95% CI)
Vit D	1.526	NA	4.600 (1.044-20.272)
Vit D + race	1.543	1.1	4.678 (1.061-20.619)
Vit D + sex	1.507	1.2	4.513 (1.022-19.921)
Vit D + age	1.272	16.6	3.568 (0.791-16.089)
Vit D + season	1.524	0.1	4.591 (1.034-20.372)
Vit D + nursing home resident	1.631	6.9	5.111 (1.158-22.551)
Vit D + liver disease	1.490	2.4	4.437 (1.004-19.605)
Vit D + renal disease	1.527	0.1	4.602 (1.038-20.414)
Vit D + heart failure	1.240	18.7	3.454 (0.762-15.650)
Vit D + malignancy	1.526	0.0	4.598 (1.043-20.269)
Vit D + COPD	1.495	2.0	4.457 (1.008-19.705)
Vit D + DM	1.560	2.2	4.759 (1.073-21.110)
Vit D + albumin	1.422	6.8	4.146 (0.932-18.443)

Abbreviations CI, confidence interval; COPD, chronic obstructive pulmonary disease; DM, diabetes mellitus; NA, not applicable; Vit D, vitamin D status; Δ, delta.

[a] The change in regression coefficients and odds ratio's produced by each potential confounder.

Appendix C

Prognostic accuracies for all predictors of 30-day mortality: individually and combined with vitamin D status

Predictors	Area under the curve (95% CI)	Regression coefficient (for vitamin D status)	p-value (for vitamin D status)
Vit D[a]	0.69 (0.57-0.80)	1.930	0.012
Cortisol	0.63 (0.51-0.76)		
Cortisol + Vit D[a]	0.74 (0.63-0.85)	1.782	0.021
CURB-65 score	0.77 (0.66-0.89)		
CURB-65 score + Vit D[a]	0.84 (0.76-0.91)	2.268	0.032
PSI score	0.78 (0.64-0.91)		
Final model:			
PSI score + Vit D[a]	0.83 (0.71-0.94)	1.832	0.021

Abbreviations: CI, confidence interval; PSI, pneumonia severity index; Vit D, vitamin D status.
[a] 2 categories of vitamin D status were analysed: 25-hydroxyvitamin D <50 nmol/L and ≥50 nmol/L.

Appendix D

Unadjusted odds ratio's for the association between vitamin D status and adverse clinical outcome (composite endpoint mortality/ICU admission)

In accordance with Leow *et al.*[10]	>50	129 (47)	4	Reference
	30-49	78 (29)	9	4.08 (1.21-13.72)
	<30	65 (24)	14	8.58 (2.70-27.31)
In accordance with Pearce *et al.*[3]	>75	50 (18)	2	Reference
	50-75	79 (29)	2	0.62 (0.09-4.57)
	25-50	98 (36)	12	3.35 (0.72-15.59)
	<25	45 (17)	11	7.77 (1.62-37.30)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; OR, odds ratio; CI, confidence interval.
Classification by Leow *et al.*[10]: 25(OH)D >50 nmol/L = sufficient; 30-49 nmol/L = deficient; <30 nmol/L = severe deficient.
Classification by Pearce *et al.*[3]: 25(OH)D >75 nmol/L = optimal; 50-75 nmol/L = adequate; 25-50 nmol/L = deficient, associated with disease risk; <25 nmol/L = deficient, associated with rickets and osteomalacia.

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The role of vitamin D supplementation in the risk of developing pneumonia: three independent case-control studies

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Submitted

Abstract

Background

The aim of this study was to examine whether vitamin D supplementation is associated with a lower pneumonia risk in adults.

Methods

Three independent case-control studies in different settings with respect to hospitalisation status. Study 1 included patients hospitalised with pneumonia who participated in two pneumonia trials in the Netherlands and controls drawn from the Dutch PHARMO Record Linkage System database. In study 2, both pneumonia cases (hospitalised patients) and controls were retrieved from the PHARMO database. Study 3 included pneumonia cases (primary care patients) and population controls, both obtained from the Netherlands Primary Care Research Network. A total of 33,726 cases and 105,243 controls were included. Cases and controls were matched by year of birth, gender and index date. Main outcome measure was exposure to vitamin D supplementation at the time of pneumonia diagnosis. Conditional logistic regression was used to compute odds ratios (OR) for the association between vitamin D supplementation and occurrence of pneumonia.

Results

Vitamin D supplementation was not associated with a lower risk of pneumonia. In study 1 and 2, adjustment for confounding resulted in non-significant ORs of 1.814 (95% CI 0.865-3.803) and 1.007 (95% CI 0.888-1.142), respectively. In study 3, after adjustment for confounding, the risk of pneumonia remained significantly higher among vitamin D users (OR 1.496, 95% CI 1.208-1.853). Additional analyses showed significant modification of the association through co-use of corticosteroids and drugs that affect bone mineralisation. For patients using these drugs, ORs below one were found combined with higher ORs for patients not using these drugs.

Conclusions

This study showed no preventive effect of vitamin D supplementation on the risk of pneumonia in adults. Alteration of the association by co-use of drugs that affect bone mineralisation and oral corticosteroids requires further study as this may reflect that effects of vitamin D supplementation depend on indication and/or underlying illnesses.

Introduction

Despite advances in its treatment throughout the years, pneumonia is still a major health-care problem causing considerable morbidity and mortality, especially in elderly.[1] Vitamin D deficiency is also very common worldwide, particularly in risk groups such as the elderly and people living distant from the equator.[2, 3] An accumulating amount of data suggests that vitamin D deficiency is associated with an increased risk of respiratory tract infections in both children and adults.[4-9] A potential mechanism underlying this association might be the immunomodulatory actions of vitamin D.[10] Next to its wellknown role in calcium and bone homeostasis, vitamin D is known to enhance innate immunity, in particular by increasing the transcription of antimicrobial peptides. Vitamin D also plays a role in acquired immunity by its effect on dendritic cells, monocytes, T-cells and B-cells.[10]

Both the increased risk of pneumonia in patients with vitamin D deficiency and the interaction between vitamin D and the immune system lead to the question whether vitamin D supplementation can lower the incidence of pneumonia. To date, several studies in both children and adults have investigated the effect of vitamin D supplementation on the incidence of respiratory tract infections, however, with conflicting findings. In one placebo-controlled trial, daily vitamin D supplementation during the winter reduced the incidence of seasonal Influenza A in schoolchildren in Japan.[11] In another randomised controlled trial, a single high dose of vitamin D for children with pneumonia reduced the risk of repeat episodes of pneumonia.[12] In contrast, three other well-designed randomised controlled trials (two conducted in adults and one in a high risk infant population) were not able to show a preventive effect of vitamin D supplementation on the incidence of respiratory tract infections.[13-15] One of these studies even reported an increased risk of repeat episodes of pneumonia for children receiving vitamin D supplementation versus placebo.[14]

Considering all this, the preventive role of vitamin D supplementation in the development of respiratory infections remains uncertain. Therefore, in this project, we conducted three independent case-control studies parallel to examine the association between vitamin D supplementation and the risk of pneumonia. In contrast with former studies including children or healthy adults, we examined the association in a relevant population at risk for pneumonia, namely elderly patients known to represent a considerable prevalence of vitamin D deficiency. Our a priori hypothesis was that vitamin D supplementation has a preventive effect on the development of pneumonia.

Methods

Study design and data origin

Three independent case-control studies were conducted comprising cases and controls from different settings with respect to hospitalisation status. Study 1 included hospitalised patients with community-acquired pneumonia who participated in two clinical trials in the Netherlands (ANTONIUS cases) and controls drawn from the PHARMO Record Linkage System database (PHARMO controls).[16, 17] The PHARMO Institute (Utrecht, the Netherlands) is an independent scientific research organisation studying drug use and outcomes. The PHARMO database includes detailed information on patient demographics, drug use and hospital admissions from about 3 million community-dwelling inhabitants of 48 geodemographic areas in the Netherlands (www.pharmo.nl). In study 2, both cases and controls were retrieved from the PHARMO Record Linkage System database (PHARMO cases and PHARMO controls). Study 3 included primary care cases and controls obtained from the Netherlands Primary Care Research Network (NPCRD) database (NPCRD cases and NPCRD controls). This nationwide database is coordinated by NIVEL, the Netherlands institute for health services research. The database is collected by a network of general practitioners across the Netherlands and includes longitudinal data on clinical diagnoses, drug prescriptions and referrals from about 700,000 individuals representative for the Dutch population.[18]

Case-control study 1 (ANTONIUS-PHARMO)

ANTONIUS cases were patients hospitalised with a radiographically confirmed community-acquired pneumonia who participated in two clinical studies.[16, 17] Patients were admitted through the emergency department of the St. Antonius Hospital in Nieuwegein or the Gelderse Vallei Hospital in Ede (both teaching hospitals in the Netherlands) between October 2004 and August 2006, and between November 2007 and September 2010. Pneumonia was defined as presence of a new pulmonary infiltrate on a chest radiograph, in combination with at least two of the following criteria: cough, sputum production, temperature above 38^{0}C or below 35^{0}C, auscultatory findings consistent with pneumonia, C-reactive protein concentration of more than 15 mg/L, white blood cell count of more than $10x10^{9}$ cells/L or lower than $4x10^{9}$ cells/L, or >10% of rods in leukocyte differentiation. On the day of hospital admission, the pneumonia severity index (PSI) and CURB-65 scores were calculated for all patients.

Population based PHARMO controls were individually matched to the ANTONIUS cases by year of birth, gender and index date, in a 4:1 ratio. The index date was the date of pneumonia diagnosis of the corresponding case. Controls were considered ineligible if they were diagnosed with pneumonia in the 6 months before the index date (identified by the International Classification of Diseases-9th revision-Clinical Modification (ICD-9-CM) codes 481-487).

Case-control study 2 (PHARMO-PHARMO)

PHARMO cases were patients hospitalised with pneumonia (both community-acquired and hospital-acquired) between 2004 and 2010. Cases were identified based on hospital discharge records coded according to the ICD-9-CM codes 481-487. Population based PHARMO controls were individually matched to the PHARMO cases by year of birth, gender and index date, in a 2:1 ratio. Controls were excluded from the study if they were diagnosed with pneumonia in the 6 months before the index date (identified by ICD codes).

Case-control study 3 (NPCRD-NPCRD)

NPCRD cases were primary care patients diagnosed with pneumonia by the general practitioner (GP) between 2004 and 2010. Diagnosis was based on clinical criteria, mainly without radiological confirmation. Cases were identified based on GP morbidity records coded by using the International Classification of Primary Care (ICPC) scheme. The ICPC code used for this purpose was R81 ('pneumonia'). Population based NPCRD controls were individually matched to the NPCRD cases by year of birth, gender and index date, in a 5:1 ratio. Controls were excluded from the study if they were diagnosed with pneumonia in the 6 months before the index date (identified by ICPC codes).

Exposure definition

For the ANTONIUS cases, community pharmacies were approached in order to identify all dispensed prescription drugs issued in the 6 months before the diagnosis of pneumonia. The PHARMO database supplied drug dispensing records for the related cases and controls. The NPCRD database provided drug prescription data for the related cases and controls. Exposure definitions were identical for all cases and controls in all three case-control studies. We identified all prescriptions for both vitamin D monotherapy and combination supplements by recording the following Anatomical Therapeutic and Chemical (ATC) codes: A11CC (vitamin D and analogues), H05BX02 (paricalcitol), A12AX (calciumcarbonate/colecalciferol), M05BB03 (alendronic acid and colecalciferol),

M05BB04 (risedronic acid, calcium, and colecalciferol, sequential), and M05BB05 (alendronic acid, calcium, and colecalciferol, sequential).
Vitamin D use was defined as two or more prescriptions in the 6 months (182 days) before the index date. Defined daily doses were calculated based on strength and prescribed dosing regimen of the most recent prescription prior to the index date.[19]

Potential confounders

Comorbidities predisposing to pneumonia were identified by drug proxies (as indicator for disease).[20] The following comorbidities were considered potential confounders: diabetes mellitus (identified by use of antidiabetics), COPD or asthma (identified by use of inhalation medication), osteoporosis (identified by use of bisphosphonates and/or calcium), advanced renal disease (identified by use of erythropoietin and/or phosphate binders), and congestive heart failure (identified by use of digoxin plus diuretics). Oral corticosteroids and enzyme-inducing anticonvulsants were also considered potential confounders because of their interaction with vitamin D metabolism.[21-23] For all drugs, except for erythropoietin and phosphate binders, drug use was defined as two or more prescriptions in the six months before the index date. Erythropoietin and phosphate binders use was defined as one or more prescriptions in the six months before the index date.

Statistical analysis

Patient characteristics were compared using the independent samples T-test, the Chi-square test or the Fisher's exact test, where appropriate. Conditional logistic regression was performed to study the association between vitamin D supplementation and the risk of pneumonia. Unadjusted odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated. Confounders were selected for the multivariate model stepwise by direct estimation of the degree of confounding produced by each factor (relative change of the regression coefficient for pneumonia associated with vitamin D supplementation). Factors that modified the regression coefficient by >10% were included in the final model to obtain adjusted ORs and 95% CIs. Additionally, we evaluated the same factors (from univariate analysis or previously mentioned in literature) as potential effect modifiers. We considered factors as an effect modifier when their interaction term (factor * vitamin D use) was statistically significant in a conditional logistic regression model with the factor and vitamin D use included. All statistical analyses were performed using SPSS 18.0 (Chicago, Illinois, USA). In all analyses, a p-value <0.05 was considered statistically significant.

Results

Patient characteristics

A total of 33,726 pneumonia cases and 105,243 matched controls were included in this project, subdivided over three independent case-control studies. Case-control study 1 comprised 504 pneumonia cases and 2,016 matched controls, case-control study 2 comprised 20,824 pneumonia cases and 41,268 matched controls, and case-control study 3 consisted of 12,398 pneumonia cases and 61,959 matched controls. The patient characteristics of cases and controls for all study cohorts are shown in Table 1. Overall, cases were more likely to have comorbid illnesses such as diabetes mellitus, congestive heart failure, osteoporosis, COPD or asthma, and more often used vitamin D supplementation and oral corticosteroids.

Association between vitamin D supplementation and pneumonia

Case-control study 1

In the crude analysis in the ANTONIUS-PHARMO cohort, vitamin D supplementation was associated with a higher risk of pneumonia (OR 3.428, 95% CI 1.906 to 6.165). The following confounders were identified: osteoporosis (58% modification of the regression coefficient), oral corticosteroids (16%) and COPD/asthma (12%). Adjustment for these confounders resulted in a non-significant risk of pneumonia among vitamin D users as compared to non-users (OR 1.814 (95% CI 0.865 to 3.803)).

Case-control study 2

In the crude analysis in the PHARMO-PHARMO cohort, vitamin D supplementation was associated with a higher risk of pneumonia (OR 1.708, 95% CI 1.556 to 1.874). In this cohort, osteoporosis (79% modification of the regression coefficient), oral corticosteroids (32%), COPD/asthma (24%) and advanced renal disease (17%) were identified as confounders. Adjustment for these confounders resulted in a non-significant risk of pneumonia among vitamin D users as compared to non-users (OR 1.007, 95% CI 0.888 to 1.142). No dose-effect relation was found in the association between vitamin D supplementation and pneumonia risk (data not shown).

Table 1. Patient characteristics for the three independent case-control studies.

	Case-control study 1		Case-control study 2		Case-control study 3	
	ANTONIUS cases (n = 504)	PHARMO controls (n = 2,016)	PHARMO cases (n = 20,824)	PHARMO controls (n = 41,268)	NPCRD cases (n = 12,398)	NPCRD controls (n = 61,959)
Age, years (SD)	63.41 (18.04)	63.00 (18.04)	66.14 (17.06)	65.98 (17.04)	60.83 (18.21)	60.73 (18.22)
Gender, men (%)	294 (58.3)	1,180 (58.5)	10,795 (51.8)	21,223 (51.4)	5,980 (48.2)	29,871 (48.2)
Quarter (%):						
January-March	172 (34.1)	NA	6,356 (30.5)	NA	4,466 (36.0)	NA
April-June	135 (26.8)		5,042 (24.4)		2,778 (22.4)	
July-September	72 (14.3)		4,055 (19.5)		1,976 (15.9)	
October-December	125 (24.8)		5,371 (25.8)		3,178 (25.6)	
Advanced renal disease (%)	4 (0.8)	8 (0.4)	**324 (1.6)**	**214 (0.5)**	43 (0.3)	79 (0.1)
CHF (%)	**21 (4.2)**	**32 (1.6)**	**654 (3.1)**	**700 (1.7)**	**170 (1.4)**	**474 (0.8)**
COPD/asthma (%)	**128 (25.5)**	**123 (6.1)**	**5,497 (26.4)**	**3,510 (8.5)**	**1,865 (15.0)**	**2,883 (4.7)**
Diabetes mellitus (%)	**77 (15.4)**	**209 (10.4)**	**2,584 (12.4)**	**3,894 (9.4)**	**974 (7.9)**	**3,455 (5.6)**
Osteoporosis (%)	**44 (8.7)**	**60 (3.0)**	**1,680 (8.1)**	**1,912 (4.6)**	**380 (3.1)**	**1,248 (2.0)**
Vitamin D supplementation (%)	**21 (4.2)**	**25 (1.2)**	**861 (4.1)**	**1,033 (2.5)**	**184 (1.5)**	**490 (0.8)**
Anticonvulsants (%)	**8 (1.6)**	**8 (0.4)**	**292 (1.4)**	**321 (0.8)**	**87 (0.7)**	**267 (0.4)**
Oral corticosteroid use (%)	**48 (9.6)**	**30 (1.5)**	**2,550 (12.2)**	**725 (1.8)**	**567 (4.6)**	**511 (0.8)**
CURB-65 score (SD)	1.69 (1.19)	NA	NA	NA	NA	NA
PSI score (SD)	89 (36)	NA	NA	NA	NA	NA
PSI risk class I-III (%)	279 (55.4)	NA	NA	NA	NA	NA
PSI risk class IV-V (%)	225 (44.6)	NA	NA	NA	NA	NA

Data are presented as number (%) or mean (±SD).
Abbreviations: CHF, congestive heart failure; NA, not applicable; PSI, Pneumonia Severity Index; SD, standard deviation.
Bold numbers indicate a significant association between cases and controls in the corresponding case-control study (p-value <0.05).

Case-control study 3
In the crude analysis in the NPCRD-NPCRD cohort, vitamin D use was associated with a higher risk of pneumonia (OR 1.905, 95% CI 1.604 to 2.262). In this cohort, osteoporosis (36% modification of the regression coefficient), COPD/asthma (21%) and oral corticosteroids (20%) were identified as confounders. Adjustment for these confounders gave a lower, but still elevated, risk of pneumonia among vitamin D users as compared to non-users (OR 1.496, 95% CI 1.208 to 1.853).

For all three case-control studies, Table 2 lists the unadjusted and adjusted ORs for the association between vitamin D supplementation and pneumonia risk. Figure 1 shows the change of the OR for vitamin D supplementation and pneumonia risk by adding each confounder stepwise.

Identification of effect modifiers

In addition to the overall analyses, the possibility of effect modification was ascertained in all three case-control studies. 'Osteoporosis' was a significant effect modifier in case-control studies 1 and 2 (p:0.018 and p:0.000, respectively), and borderline significant in case-control study 3 (p:0.072). 'Oral corticosteroids' was a significant effect modifier in case-control study 2 (p:0.024) and 3 (p:0.006), but not in case-control study 1 (p:0.121). Adjusted ORs of these effect modifiers can be found in Table 3.

Table 2. Unadjusted and adjusted odds ratio's for the association between vitamin D supplementation and pneumonia risk for the three independent case-control studies.

		Cases	Controls	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Case-control study 1	Subjects	504	2016		
ANTONIUS-PHARMO	No vit D use	475 (95.8)	1,991 (98.8)	Reference	Reference
	Vit D use	21 (4.2)	25 (1.2)	3.428 (1.906-6.165)	1.814† (0.865-3.803)
Case-control study 2	Subjects	20,824	41,268		
PHARMO-PHARMO	No vit D use	19,963 (95.9)	40,235 (97.5)	Reference	Reference
	Vit D use	861 (4.1)	1,033 (2.5)	1.704 (1.552-1.871)	1.007‡ (0.888-1.142)
Case-control study 3	Subjects	12,398	61,959		
NPCRD-NPCRD	No vit D use	12,214 (98.5)	61,469 (99.2)	Reference	Reference
	Vit D use	184 (1.5)	490 (0.8)	1.905 (1.604-2.262)	1.496† (1.208-1.853)

Data are presented as number (%), unless otherwise indicated. Abbreviations: CI, confidence interval; OR, odds ratio; VitD, vitamin D use. † OR adjusted for osteoporosis, COPD/asthma and oral corticosteroid use ‡ OR adjusted for osteoporosis, COPD/asthma, oral corticosteroid use and advanced renal disease.

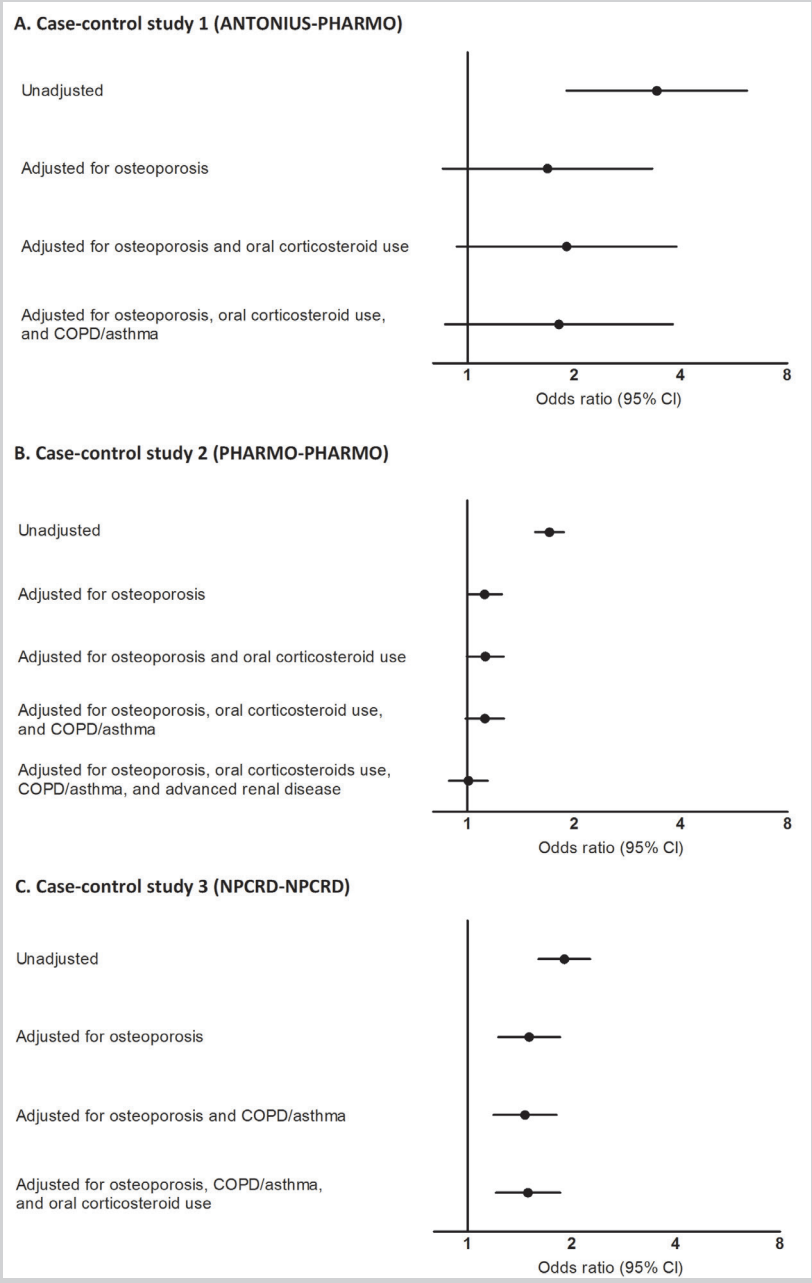

Figure 1. Odds ratios on a log scale in the association between vitamin D use and pneumonia with progressive multivariate adjustment for confounders in three independent case-control studies.

Table 3. Effect modification of adjusted odds ratios for the risk on pneumonia with vitamin D supplementation in all three case-control studies.

	Case-control study 1 ANTONIUS-PHARMO	Case-control study 2 PHARMO-PHARMO	Case-control study 3 NPCRD-NPCRD
Vitamin D use	1.814 (0.865-3.803)†	1.007 (0.888-1.142)‡	1.496 (1.208-1.853)†
Vitamin D use wtihout osteoporosis	2.631 (1.243-5.566)†	1.416 (1.132-1.772)‡	1.671 (1.124-2.486)†
Vitamin D use with osteoporosis	1.011 (0.536-1.907)†	0.892 (0.746-1.066)‡	1.432 (1.113-1.843)†
Vitamin D use without oral corticosteroid use	1.716 (0.977-3.016)†	1.028 (0.902-1.172)‡	1.605 (1.288-2.000)†
Vitamin D use with oral corticosteroid use	0.826 (0.316-2.163)†	0.854 (0.625-1.168)‡	0.817 (0.463-1.442)†

Data are presented as odds ratio (95% confidence interval).
† Odds ratio adjusted for osteoporosis, COPD/asthma and oral corticosteroid use. ‡ Odds ratio adjusted for osteoporosis, COPD/asthma, oral corticosteroid use and advanced renal disease.

Discussion

In three independent case-control studies in adults, vitamin D supplementation was not associated with a lower risk of developing pneumonia. In primary care setting, an increased pneumonia risk was observed for vitamin D users. Interestingly, the use of drugs that affect bone mineralisation (bisphosphonates and/or calcium) or oral corticosteroids significantly modified the association.

The absence of a preventive association between vitamin D supplementation and pneumonia was contrary to our expectations. This finding, however, is in accordance with the most recent placebo-controlled studies on vitamin D and respiratory tract infections. In 3,000 infants in Afghanistan a quarterly dose of 100,000 IU vitamin D3 or placebo during 18 months did not affect the incidence of pneumonia. Interestingly, they even showed a significant higher incidence rate of repeat episodes of pneumonia in vitamin D treated children.[14] The recent placebo-controlled study by Murdoch *et al*. also showed no reduction in the incidence or severity of upper respiratory tract infections in 322 healthy adults in New Zealand who randomly received oral vitamin D_3 (an initial dose of 200,000 IU followed by 200,000 IU after 1 month, and then 100,000 IU monthly for 18 months) or placebo.[15] The most recent study by Li-Ng *et al*. conducted in 162 adult volunteers from Long Island, New York, who were randomised to either 2,000 IU vitamin D3 daily for 12 weeks or placebo during the winter, also reported no benefit of vitamin D supplementation in decreasing the incidence of upper respiratory tract infections.[13] In our study, including more than 130,000 mostly elderly persons (comprising 4,432 vitamin D users) also no association between vitamin D supplementation and risk of acquiring pneumonia was observed. This suggests that any preventive effects, if present, also do not extend to the elderly, who are known to represent a considerable prevalence of vitamin D deficiency.[24] So far, studies in which a preventive effect of vitamin D supplementation on respiratory tract infections was found remain studies conducted in children.[11, 12] Maybe, future studies including genetic data will be able to identify small subgroups that might benefit from vitamin D supplementation.[25]

The modification of the association between vitamin D supplementation and pneumonia by drugs that affect bone mineralisation ('osteoporosis') and oral corticosteroids may have different explanations. First, there might be a pharmacological interaction between vitamin D and these drugs.

An interaction between vitamin D and calcium has been suggested in a recent individual patient data meta-analysis that showed a reduced overall mortality

in elderly patients using vitamin D and calcium together, whereas sole use of vitamin D supplementation did not affect the mortality rate.[26, 27] This could mean that co-use of calcium is necessary for vitamin D to become beneficial. Second, it might also be that in patients with osteoporosis vitamin D will most likely be prescribed for its effect on calcium metabolism and not because of confirmed vitamin D deficiency. It is not inconceivable that the vitamin D dosages observed in our study are insufficient to overcome true vitamin D deficiency and thus explain why vitamin D supplementation in patients not using osteoporosis drugs is associated with an increased pneumonia risk. Linked to the latter, it is also possible that in patients not using drugs that affect bone mineralisation, vitamin D is prescribed for indications that predispose for pneumonia but that those indications were not available for assessment as confounder in the present study. The percentage of vitamin D users that also used bone mineralisation affecting drugs varied between 72 and 80% across the three datasets. In study 3, after adjustment for confounding, there remained overall a significant increased risk of pneumonia in patients receiving vitamin D supplementation. Nevertheless, the effect modification was similar in all three datasets indicating no preventive effect of vitamin D supplementation on pneumonia risk in patients using vitamin D adjacent to drugs that affect bone mineralisation.

Major strengths of the present project are the very large numbers of patients, extensive adjustment for confounding, and the fact that we studied the same association in three cohorts in different settings with respect to pneumonia diagnosis and hospitalisation status. The fact that comparable results were observed adds to the robustness of the findings. Furthermore, the three studies were conducted in the same country and time period, limiting the possibility of differences in medical guidelines, insurance policy or social-economic situation as sources of heterogeneity.

There are limitations to our project that need to be addressed. First, the absence of an association might be caused by residual confounding as a result of missing complete information on medical diagnoses. Proxies, varying in robustness, were used as indicators for comorbidity and therefore only patients treated with medication were recognized as having a comorbid illness. Second, data about over-the-counter vitamin D use were not available, something which might have lead to an underestimation of the true effects of vitamin D supplementation on pneumonia risk. Third, it was not possible to adjust for baseline vitamin D status because 25-hydroxyvitamin D levels in blood were

not available. Possibly, vitamin D was not supplemented adequately enough to correct vitamin D status to normal. It cannot be ruled out that for prevention of pneumonia larger amounts of vitamin D are needed to acquire an effect. The small number of patients receiving high-dose vitamin D supplements in the present study precluded a thorough analysis of dose dependency. On the other hand, the present project also did not show a trend towards any dose dependency. Finally, we were not able to verify pneumonia diagnosis in all studies. In study 1 all pneumonia diagnoses were radiographically confirmed, but in study 2 and 3 patients were selected in administrative databases. However, the patient characteristics of study 2 resemble the patient characteristics of study 1 very much and several studies have shown high positive values for pneumonia coding in administrative databases.[28, 29]

In conclusion, three large independent case-control studies showed no preventive association between vitamin D supplementation and pneumonia. In patients using vitamin D supplementation without drugs that affect bone mineralisation or oral corticosteroids, the risk of pneumonia was increased. This might indicate unmeasured confounding, insufficient supplementation or a true risk. Further research is necessary to explore the interaction between vitamin D supplementation and concurrent drugs on the risk of developing pneumonia.

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Summary, general discussion and perspectives

Summary

Community-acquired pneumonia (CAP) is a common disease, which causes considerable morbidity and mortality worldwide. Despite the availability of effective antibiotics, pneumonia remains the leading cause of death from infectious diseases and mortality from CAP has not decreased in the last decades. To further improve outcome of lower respiratory tract infections, new (non-antibiotic) treatment strategies are therefore urgently needed. In this thesis, options for adjuvant treatment are described, with a focus on immunomodulation.

The immune response in community-acquired pneumonia

The host response to pneumonia is characterised by an acute inflammatory response. The nature of the inflammatory response to a degree is determined by the type of microorganism that is encountered. Thus, intracellular bacteria require a different response from the immune system than extracellular bacteria. The existing knowledge on pathogen-specific inflammatory response patterns in CAP however is limited, and therefore, further elucidation of pathogen-specific inflammatory profiles is needed.

In **Chapter 2**, we report the measurement of a comprehensive panel of systemic inflammatory markers in 469 patients with CAP, including prototype pro- and anti-inflammatory cytokines and chemokines. Compared to patients with CAP caused by an atypical pathogen or a virus, patients with pneumococcal pneumonia showed significantly higher leukocyte counts, procalcitonin (PCT), interleukin-6 (IL-6), interleukin-1 receptor-antagonist (IL-1ra) and monocyte-chemotactic-protein-1(MCP-1) concentrations. The PCT concentration was comparable between those with atypical pathogens and viruses. Patients with CAP caused by an atypical pathogen had significantly higher IL-17 and interferon-γ (IFN-γ) concentrations than did patients with CAP caused by other micro organisms. Moreover, patients with an atypical pathogen had the lowest concentrations of IL-10 and leukocyte counts. Within the group of atypical pathogens, also differences in the inflammatory response were found. In particular, *Legionella pneumophila* elicited the highest concentrations of C-reactive protein (CRP), PCT, leukocytes, IL-6, tumour necrosis factor-α (TNF-α), IL-10 and IL-8. Antibiotic use prior to admission did not change inflammatory profiles. We conclude that the major causative microorganisms in CAP trigger distinct inflammatory response profiles in the host.

While an inflammatory response as such is required to combat invading

pathogens, an excessive inflammatory response may contribute to adverse outcome. Modulation of the immune response could therefore offer promising treatment options in CAP. In this thesis, we focus on the immunomodulatory properties of corticosteroids, macrolides and vitamin D, and their potential role in prevention and treatment of CAP.

Immunomodulation by dexamethasone

Corticosteroids are potentially of use for adjuvant treatment in CAP due to their dampening effect on the inflammatory response and/or reversal of an inadequate hypothalamic-pituitary-adrenal (HPA) axis response. Adjuvant corticosteroids have already been proven beneficial in other infectious diseases, such as bacterial meningitis and septic shock. The effectiveness of corticosteroids in CAP is still a matter of debate because prior studies in CAP have reported conflicting results.

In a randomised placebo-controlled trial, we studied the effect of adjuvant dexamethasone on length of hospital stay in non-immunocompromised adults with CAP. The results of this trial are shown in **Chapter 3**. On hospital admission with CAP, patients were randomly assigned to either a four-day course of adjuvant dexamethasone (5 mg once a day) or placebo. A total of 304 patients were included, of which 151 received dexamethasone and 153 received placebo. Compared with placebo, adjuvant dexamethasone reduced length of hospital stay by one day (median length of stay 6.5 days versus 7.5 days; p:0.048). Furthermore, levels of CRP and IL-6 decreased faster in the dexamethasone group than in the placebo group, while the decline in IL-10 levels did not differ between the two groups. Hyperglycaemia was more often detected in the dexamethasone group, but severe adverse events were rare and did not differ between the groups.

The influence of dexamethasone on the cytokine response in CAP is further explored in **Chapter 4**. In serum from the aforementioned 304 patients, an extensive panel of cytokines and chemokines was measured. In general, compared to placebo-treated patients, dexamethasone-treated patients had a faster decline of IL-6, IL-8, MCP-1 and TNF-α. Differences in dexamethasone effect were found for different microbial aetiologies. In patients with pneumococcal pneumonia, dexamethasone had little additional influence on cytokine concentrations, while in patients with CAP caused by an atypical pathogen, dexamethasone gave a faster decrease of the levels of IL-1ra, IL-6 and MCP-1.

The beneficial effect of exogenous corticosteroids suggests that in CAP the physiological regulatory systems for the inflammatory response are

inadequate. Cortisol, the endogenous corticosteroid secreted by the adrenal cortex, is an important regulator of inflammation and exerts anti-inflammatory and immunosuppressive activities. Generally, severe illness and stress strongly activate the HPA-axis, leading to an increased level of cortisol. However, many critically ill patients develop dysfunction of the HPA-axis. This syndrome is referred to as critical illness-related corticosteroid insufficiency (CIRCI), defined as cortisol <10 µg/dL or a change in serum cortisol (delta) of <9 µg/dL after administration of 250 µg synthetic ACTH. CIRCI is associated with poor outcome and corticosteroid replacement may be beneficial in these patients. In **Chapter 5**, we investigated whether subgroups can be defined that will benefit in particular from corticosteroid therapy, based on the cortisol response to infection. In patients with CAP, we showed that a low serum cortisol <10 µg/dL was not associated with adverse clinical outcome. Most of the patients with a serum cortisol <10 µg/dL had non-severe disease rather than corticosteroid insufficiency. This finding suggests that the current definition of CIRCI is not applicable in patients with CAP, as low cortisol responses merely reflect low disease severity in many patients. We show that patients with a high pro-inflammatory cytokine response but a discrepantly low cortisol benefited most from adjuvant dexamethasone. In the placebo group, 43% of the patients died or were admitted to the ICU, compared to 0% in the dexamethasone group (p:0.020). We conclude that in CAP patients, the relation between cytokine response and cortisol level might better reflect corticosteroid insufficiency and might help to identify patients who may benefit in particular from adjuvant corticosteroid therapy.

A high cortisol level on admission with CAP is known to be associated with adverse clinical outcome. Whether this also holds true for cortisol levels that remain increased during the course of disease is unknown. Moreover, a potential risk of synthetic corticosteroids is secondary adrenal insufficiency. The time to recovery of the HPA axis after a short course of dexamethasone during infection is unclear. In **Chapter 6**, we report serial cortisol measurements throughout the course of CAP. It is shown that persisting high cortisol levels are also associated with poor outcome. In addition to cortisol level on admission, which may serve as prognostic biomarker in CAP, the change in cortisol from day zero to day two or four could be another meaningful biomarker in CAP. Additionally, we report that the endogenous cortisol production was almost completely suppressed by dexamethasone therapy after the first dose, but was fully recovered on day 30.

Immunomodulation by macrolides

Macrolides are known to possess immunomodulatory properties next to their antimicrobial effects. The immunomodulatory effects of macrolides are beneficial in chronic pulmonary inflammatory syndromes, such as diffuse panbronchiolitis, cystic fibrosis, asthma and bronchiectasis. Whether macrolides also exert favourable immunomodulatory effects during acute inflammatory conditions, such as CAP, is less clear.

In **Chapter 7**, we provide an overview of the existing evidence from *in vitro* and *in vivo* studies on the immunomodulatory effects of macrolides in CAP. Macrolides were shown to change the nature of the immune response during acute inflammation in three ways: by suppression of the cytokine response, by changing the behaviour of inflammatory cells to a more anti-inflammatory nature and by affecting structural cells of the respiratory tract. The existing evidence is restricted by heterogeneity of the studies, with regard to methods and experimental model systems. Furthermore, experimental and clinical studies on the immunomodulatory effects of macrolides when given adjuvant to β-lactam antibiotics, are lacking.

In order to further elucidate the mechanisms of immunomodulation by macrolides during acute inflammation, in particular when given in combination with β-lactam antibiotics, we designed an *in vitro* model of acute infection with *Streptococcus pneumoniae*. In **Chapter 8**, we report that macrolides, alone or adjuvant to β-lactam antibiotics, attenuated the pro-inflammatory cytokine response in whole blood stimulated with heat-killed *S. pneumoniae*. This suggests an immunomodulatory effect of macrolides. This effect was not observed in experiments with viable *S. pneumoniae* (either macrolide-susceptible or -resistant). Stimulation of whole blood with viable pneumococci induced an overwhelming cytokine response, but no difference in cytokine response was detected between a macrolide-containing antibiotic regimen and β-lactams alone.

Immunomodulation by vitamin D

Vitamin D has pleiotropic immunomodulatory properties apart from its function in calcium and bone homeostasis. Vitamin D deficiency, which is common worldwide, is associated with an increased susceptibility to respiratory tract infections. In patients hospitalised with CAP, vitamin D deficiency has been associated with adverse clinical outcome, but the prognostic value of vitamin D status is unknown. In **Chapter 9**, it is reported that in our CAP cohort, 53% of

the patients was vitamin D deficient. We confirmed that vitamin D deficiency is associated with adverse clinical outcome in CAP. Median 25-hydroxyvitamin D levels were significantly lower in patients who were admitted to the intensive care unit (ICU), compared with patients without ICU admission (34.9 nmol/L versus 48.3 nmol/L; p:0.04). Patients who died within 30 days had significantly lower 25-hydroxyvitamin D levels, compared with patients who survived (25.8 nmol/L versus 48.8 nmol/L; $p<0.01$). Vitamin D status at the time of hospital admission appeared to be an independent predictor for 30-day mortality, adding prognostic value to other biomarkers and prognostic scores. With an area under the curve (AUC) of 0.83, the prognostic accuracy of the combination of vitamin D status and PSI score was significantly superior to the accuracy of other predictors of 30-day mortality, or the PSI score alone.

Whether the association between vitamin D deficiency and increased susceptibility to infections, and the association between vitamin D deficiency and poor outcome in CAP, are based on a causal relationship is unknown. In that case, vitamin D supplementation to high risk populations or in the acute care of CAP would be beneficial. Therefore, in three large independent case control studies described in **Chapter 10**, we studied the association between vitamin D supplementation and the risk of pneumonia. In hospitalised patients in study 1 and 2, after adjustment for confounding, vitamin D supplementation was not associated with a lower risk of developing pneumonia. In a primary care setting in study 3, after adjustment for confounding, even an increased risk of pneumonia was observed among vitamin D users. Interestingly, the use of bisphosphonates and/or calcium or oral corticosteroids significantly modified the association, suggesting that effects of vitamin D supplementation depend on indication and/or underlying illnesses. In patients using vitamin D supplementation without concomitant use of bisphosphonates, calcium or oral corticosteroids, the risk of pneumonia was increased. In conclusion, based on the current available data, (standard) vitamin D supplementation cannot be recommended for prevention of CAP.

General discussion and perspectives

Before the antibiotic era, pneumonia was considered 'The Captain of the Men of Death'.[1] Mortality rates among non-bacteremic patients with pneumococcal pneumonia ranged from about 8% in the youngest group to over 80% in elderly, whereas, among bacteremic patients, mortality ranged from 28% in the youngest group to 100% in elderly.[2] The introduction of antibacterial drugs in the late 1930s has led to a considerable decrease in mortality.[1] In the last decades, however, the availability of potent antibiotics has not resulted in a further reduction of mortality. Instead, management of pneumonia has been complicated by the emergence of antibiotic resistance. Therefore, new treatment strategies are needed to further improve outcome.

A promising option for new therapies is modulation of the immune response. An adequate immune response is pivotal for host defence against invading pathogens. When excessive, however, the immune response can cause local tissue injury to the lungs, which may lead to acute respiratory distress syndrome (ARDS), sepsis, multi-organ failure or even death. Prior studies in community-acquired pneumonia (CAP) have indeed shown that clinical outcome is related to the extent of the inflammatory response.[3, 4] Why the evolution of the immune system has permitted the immune response to become so powerful that it may even have harmful or lethal consequences is unknown. An attractive hypothesis is that the delicate balance between the immune response needed to eliminate the infection and systemic side effects of this response has been shifted by the introduction of antibiotic treatment two or three generations ago. The assistance of antibiotics in clearing of infection may have resulted in a decreased need for an inflammatory response to be mounted by the body himself. Furthermore, lytic properties of certain antibiotics may even cause a more intense inflammatory response than is essentially needed. Following this line of arguments, the introduction of antibiotics may have created a niche for immunomodulatory drugs as adjuvant treatment in CAP.[5]

The studies in this thesis mainly focus on the immunomodulatory properties of corticosteroids, macrolides and vitamin D, and their potential role in prevention and/or treatment of CAP. In this general discussion, the implications of the presented studies and the perspectives for future research are discussed.

Immunomodulation by corticosteroids

Corticosteroids are potent inhibitors of the inflammatory response.[6] Adjuvant corticosteroids have been proven beneficial in a variety of infectious diseases, such as tuberculous and bacterial meningitis.[7-9] Also in CAP, corticosteroids are thought to be promising agents for adjuvant treatment strategies, because of their immunosuppressive effects and the ability to restore steroid levels in case of (relative) adrenal insufficiency.[10]
Experimental studies indeed point towards a beneficial effect of corticosteroids in CAP. In a model of severe pneumonia in piglets, adjuvant glucocorticoid therapy was demonstrated to attenuate the local inflammatory response by decreasing the concentration of interleukin-6 (IL-6) and to decrease the bacterial burden in the lung.[11] In a mouse pneumonia model, hydrocortisone reduced vascular lymphocytes, inflammatory cytokines and nitric oxide levels (near-) significantly.[12] In human pneumonia studies focussing on the inflammatory response, corticosteroids have been shown to reduce local and systemic inflammatory responses.[13, 14]

Whether the administration of adjuvant corticosteroids in CAP leads to improved outcome has been studied in several randomised controlled trials (RCTs), seven in total (Table 1).[15-21] In some, but not all trials, a beneficial effect of corticosteroids was found. This might be due to the heterogeneity of the studies. A broad range of primary and secondary endpoints has been investigated, limiting the comparability of the studies. Next, disease severity was highly variable across the studies, and many different treatment regimens (including different types of corticosteroids) were used. Several studies included only small numbers of patients, making it difficult to prove or rule out an effect on clinically relevant endpoints. Thus, additional well-designed studies are required to ultimately draw a firm conclusion with regard to the effectiveness of corticosteroids in CAP. Therefore, in Chapter 3 of this thesis, we present the largest RCT up to now. In 304 hospitalised, non-immunocompromised patients with CAP, the administration of dexamethasone adjuvant to antibiotic treatment reduced length of hospital stay by one day. Four other trials have investigated length of stay as primary or secondary endpoint.[17-19, 21] Our finding is in line with the study by Confalonieri *et al.* in ICU patients with severe CAP, who found a significant reduction in length of stay from 21 days in the placebo-group to 13 days in hydrocortisone-group (p:0.03).[17] The other three trials did not observe a favourable effect of corticosteroids on length of hospital stay.[18,

[19, 21] However, the low number of patients enrolled in two of these trials (31 and 56 patients) probably precluded the finding of significant results.[18, 21] The large trial by Snijders *et al*., including 213 CAP patients from which 22 patients were primarily admitted to the ICU, in the first instance found a non-significant reduction in length of stay.[19] Interestingly, re-analysis revealed that, when excluding patients who were directly admitted to the ICU (in accordance with our study protocol), adjuvant prednisolone did have a borderline significant impact on length of stay.[22] Thus, taking all studies together, the scale seems to be tipping towards a beneficial effect of corticosteroids on length of stay in hospitalised, non-immunocompromised patients with CAP.

In our study, adjuvant dexamethasone treatment did not reduce the mortality rate of CAP. In the placebo arm of our study, the overall 30-day mortality was 7%, while in the dexamethasone arm this figure was 6% (p:0.68). A recent meta-analysis on corticosteroids in the treatment of CAP, which included data on mortality from eight trials, reported that mortality was not significantly reduced by the use of corticosteroids.[23] In the subgroup of patients with severe CAP, however, they observed a significant decrease in mortality associated with the use of corticosteroids. Additional, adequately powered RCTs or meta-analyses on patient-level are needed to confirm this finding.

Despite the fact that we have shown an overall beneficial effect of corticosteroids on length of hospital stay in CAP, it is possible that certain subgroups benefit more from adjuvant dexamethasone therapy and thus a stronger effect can be found in these patients, while other subgroups may have no benefit at all. Hypothetically, in patients with a low pro-inflammatory cytokine response or a sufficient pro-inflammatory cytokine response to overcome the infection by itself, corticosteroid therapy might give too much suppression of the inflammatory response, which can be harmful. Moreover, patients with relative or critical-illness related corticosteroid insufficiency may benefit in particular from corticosteroid therapy, due to steroid replenishment. In a *post-hoc* analysis of our clinical study (presented in Chapter 5), we divided patients into four subgroups based on cytokine response and cortisol level on hospital admission. We observed that in patients with a high pro-inflammatory cytokine response but a discrepantly low cortisol level, adjuvant dexamethasone gives a significant reduction on the combined endpoint of mortality or ICU admission. This correlation between cytokine response and cortisol level may

Table 1. Overview of randomised clinical trials on the effectiveness of corticosteroids in community-acquired pneumonia

Author	Year	No. of patients	Disease severity	Corticosteroid regimen	Primary endpoint	Outcome	Secondary endpoint	Outcome
McHardy *et al.*[15]	1972	126	Mild to severe (but not 'desperately ill')	Prednisolone, 20 mg/d, 7 days	Rate of recovery (including number of deaths, duration of treatment, change of treatment, resolution of temperature, clearance of initial pathogens, and maximum radiological clearing)	No benefit: Only a trend towards becoming afebrile more quickly		
Marik *et al.*[16]	1993	30	Severe	Hydrocortisone, 10 mg/kg, 1 day	Clinical course; TNF-α levels	No benefit		
Confalonieri *et al.*[17]	2005	48	Severe CAP (ICU patients)	Hydrocortisone, 240 mg/d, 7 days	Improvement of PaO2:FiO2; MODS score; reduction of delayed septic shock	Benefit: Significant improvement of PaO2:FiO2, of MODS score and significant reduction of delayed septic shock	Duration of mechanical ventilation; length of ICU/RIU and hospital stay; survival to hospital discharge and to 60 days	Benefit: Significant reduction in duration of mechanical ventilation, length of ICU/RIU and hospital stay, and increased survival to hospital discharge and to 60 days
Mikami *et al.*[18]	2007	31	PSI class I-V	Prednisolone, 40 mg, 3 days	Length of hospital stay	No benefit	Duration of IV antibiotic treatment; time required to stabilize vital signs	Benefit: Shorter duration IV antibiotics, earlier stabilisation of vital signs
Snijders *et al.*[19]	2010	213	PSI class I-V	Prednisolone, 40 mg, 7 days	Clinical cure at day 7	No benefit	Clinical cure at day 30, length of stay, time to clinical stability, defervescence and CRP	No benefit: Late failure was significantly more common among prednisolone-treated patients

Table 1. *Continued*

Author	Year	No. of patients	Disease severity	Corticosteroid regimen	Primary endpoint	Outcome	Secondary endpoint	Outcome
Sabry *et al.*[20]	2011	80	Severe	Hydrocortisone, 12.5 mg/h, 7 days	Improvement of PaO2:FiO2; SOFA score by study day 8; development of delayed septic shock	Benefit: Significant improvement in PaO2:FiO2, significant reduction in SOFA score and delayed septic shock	Chest radiograph score, CRP levels	Benefit: Significant improvement chest radiograph score, significant decline CRP levels
Fernandez-Serrano *et al.*[21]	2011	56	PSI class II-V	Methylprednisolone, 620 mg, 9 days	Need for mechanical ventilation	Trend towards reduced need for mechanical ventilation, but non-significant	pO2/FiO2 ratio, radiological improvement, TRM score, length of hospital stay, length of ICU stay, mortality, levels of systemic inflammatory response	Benefit: Improved oxygenation, faster decrease of fever, greater radiological improvement
Meijvis *et al.*[52]	2011	304	PSI class I-V	Dexamethasone, 5 mg, 4 days	Length of hospital stay	Benefit: Significant reduction of length of stay (p:0.048)	Mortality, admission to ICU, empyema, superinfection, readmission, time courses of CRP, interleukin-6 and interleukin-10 concentrations, pulmonary function at day 30, quality of life	Benefit: Significantly better quality of life with respect to social functioning by day 30

Abbreviations: CAP, community-acquired pneumonia; PSI, pneumonia severity index; ICU, intensive care unit; TNF-α, tumour necrosis factor-α; MODS, multiple organ dysfunction syndrome; SOFA, sequential organ failure assessment score; RIU, respiratory intermediate unit; IV, intravenous; CRP, C-reactive protein; TRM score, time to resolution of morbidity score.

help to identify patients who will benefit most from dexamethasone treatment. However, it must be emphasized that this conclusion is preliminary, due to the fact that this was a *post-hoc* analysis of a study that was not powered on these endpoints. The ultimate study design to confirm our findings would be a sufficiently powered RCT, in which randomisation to either dexamethasone or placebo is based on cytokine and cortisol measurements upon admission. Unfortunately, this concept is not feasible yet, since there is no measure of inflammation by cytokines that has been calibrated for use in clinical practice thus far. In our study, replacement of cytokine measurements by the concentration of C-reactive protein on presentation gave inferior results. The use of a clinical prediction rule, such as the Pneumonia Severity Index (PSI) score, as a substitute for cytokine measurements is questionable. The PSI score, which predicts 30-day mortality from CAP, is largely based upon age and comorbidities and does not necessarily reflect the intensity of the inflammatory response. This is underlined by our study population, in which only 52% of the patients with a high cytokine response had a CAP ranked as PSI class IV or V. Validation of cytokine measurements for clinical practice is therefore highly desirable.

Future research on the role of corticosteroids as adjuvant treatment in CAP should first focus on confirmation of our results in large trials. These studies should be adequately powered to enable subgroup analyses. We are looking forward to the results of three ongoing placebo-controlled trials on corticosteroids as an adjunct to antibiotic therapy: the 'STEP trial' in Switzerland, aiming to enrol 800 hospitalised patients with CAP (http://clinicaltrials.gov NCT 00973154); one study in Spain, aiming to enrol 120 patients with severe CAP (PSI class V) (http://clinicaltrials.gov NCT 00908713); and the 'Santeon-CAP' study, aiming to enrol 600 hospitalised patients with CAP (http://clinicaltrials.gov NCT 01743755). The latter study is the follow-up study of the trial presented in this thesis. When the benefit of corticosteroids can be confirmed, subsequent research should be aimed at the identification of subgroups of patients who will benefit most from corticosteroids, ideally without negative side-effects.

Immunomodulation by macrolides

Macrolides are drugs with a macrocyclic lactone ring of 12 of more elements. The 14-, 15- and 16-membered macrolides are a widely used family of antibiotics.[24] Macrolides also have non-antimicrobial, immunomodulatory properties which have been demonstrated to be effective in chronic pulmonary inflammatory

syndromes, such as diffuse panbronchiolitis, cystic fibrosis, asthma and bronchiectasis. In these chronic diseases, administration of macrolides is associated with reduced disease severity, length of hospital stay and mortality.[25] Whether these favourable effects of macrolides can be extended to acute inflammation, such as in CAP, is less clear. As reported in Chapter 7, the available literature on this subject suggests that macrolides can temper the inflammatory response during CAP. However, because the studies differ in their methodology, no definite conclusions can be drawn.

In the series of experiments presented in Chapter 8, we aimed to explore the influence of macrolides on the cytokine response in acute inflammation. In CAP, empirical treatment generally contains a β-lactam antibiotic targeted at *S. pneumoniae,* the most frequently identified micro organism. β-lactam antibiotics are cell wall active agents that cause lysis of bacteria, leading to the release of an array of bacterial components at the site of infection. Hence, intensification of the pro-inflammatory immune response may occur. For that reason, in our experiments, we were particularly interested in whether adjuvant macrolides can attenuate the excessive immunostimulation caused by β-lactam therapy. We used an *in vitro* model of acute infection with *S. pneumoniae.* In a first series of experiments, whole blood was stimulated with heat-killed *S. pneumoniae,* in order to separate possible immunomodulatory effects from direct antibacterial effects. Pre-incubation with a macrolide-containing regimen resulted in a trend towards lower levels of pro-inflammatory cytokines, compared to β-lactam mono-therapy, suggesting an immunomodulatory effect. This effect was not observed in subsequent series of experiments with viable *S. pneumoniae* (either macrolide-susceptible or -resistant), while this condition mostly resembles *in vivo* infection. The absence of an effect might be due to shortcomings in the experimental design, such as a too large inoculum of bacteria or an inappropriate incubation time. However, it might also indicate that there is a true absence of immunomodulatory effects on the cytokine response in acute inflammation. The present series of experiments only examined a single macrolide, at a single dose, at a single time point, with a single bacterial inoculum and a limited number of *S. pneumoniae* strains. The findings should therefore be considered hypothesis-generating rather than definitive. Future experiments should be extended with various bacterial inoculums, incubation times, treatment regimens and should ideally use isogenic strains of *S. pneumoniae*, in order to conclude whether adjuvant macrolides have a favourable effect on the cytokine response during acute inflammation or not.

The clinical impact of adjuvant macrolides in patients with CAP has also not been proven unequivocally to date. Several retrospective and non-randomised prospective trials have demonstrated survival benefits in patients with (pneumococcal) pneumonia treated with a combination of β-lactam antibiotics and macrolides, compared to patients treated with β-lactam monotherapy.[26-33] However, other studies have shown no difference between these two treatment regimens.[34, 35]Randomised controlled trials on this subject are urgently needed to definitively assess the clinical impact of adjuvant macrolides in CAP.
We are aware of two ongoing trials on this subject: 1. the cluster-randomised, multi-centre 'CAP-START study' in the Netherlands, which compares β-lactam monotherapy, β-lactam in combination with a macrolide, and quinolone monotherapy as empirical treatment in CAP (http://clinicaltrials.gov NCT 01660204); 2. the randomised, non-inferiority, open 'BICAP trial' in Switzerland, which compares β-lactam monotherapy with a combination of β-lactam antibiotics and macrolides in non-severe hospitalised CAP (http://clinicaltrials.gov NCT 00818610). In anticipation of the results of these trials, the potential benefit of adjuvant macrolide therapy should be weighed against the disadvantages of these agents, such as progression of antibiotic resistance. From the perspective of antibiotic resistance, the development of macrolide derivatives with anti-inflammatory activity, but without antibacterial effects is highly desirable. 'EM900' has been suggested to be such a non-antibiotic macrolide and is currently being studied in Japan.[36]

Immunomodulation by vitamin D

Vitamin D is well-known for its function in calcium and bone homeostasis. The vitamin D receptor is present in most tissues and cells in the body, including immune cells, which suggests that vitamin D is involved in many processes other than calcium and bone homeostasis. In recent years, vitamin D has been recognised to have pleiotropic immunomodulatory properties. Vitamin D deficiency, which is very common worldwide, results in abnormalities of calcium, phosphorus and bone metabolism, but is also associated with autoimmunity and increased susceptibility to infection.[37]

Specifically, vitamin D deficiency has been associated with an elevated risk of respiratory tract infections.[38-42] This association leads to the question whether vitamin D supplementation can lower the incidence of pneumonia. Prior studies investigating the preventive effect of vitamin D supplementation on the development of pneumonia have been inconclusive. Two randomised

controlled trials, both in children, did show a preventive effect of vitamin D supplementation. In one placebo-controlled trial, daily vitamin D supplementation during the winter reduced the incidence of seasonal Influenza A in schoolchildren in Japan.[43] In another trial, a single high dose of vitamin D for children with pneumonia reduced the risk of repeat episodes of pneumonia.[44] On the contrary, three well-designed randomised controlled trials (two conducted in adults and one in a high risk infant population) were not able to show a preventive effect of vitamin D supplementation on the incidence of respiratory tract infections.[45-47] One of these studies even reported an increased risk of pneumonia in patients receiving vitamin D supplementation.[46] Considering this all, the preventive role of vitamin D supplementation in the development of respiratory tract infections remains uncertain. Therefore, in Chapter 10, we conducted three independent case-control studies parallel to examine the association between vitamin D supplementation and the risk of pneumonia in adults. We found that vitamin D supplementation was not associated with a lower risk of developing pneumonia. This finding suggests that there is no causal relation between vitamin D deficiency and the risk of pneumonia, and that vitamin D deficiency is just a marker of frailty and poor prognosis. Nevertheless, it is also possible that a causal relation does exist, but remained undetected in our study. Despite our attempt to thoroughly adjust for confounders, there might have been residual confounding as a result of missing complete information on medical diagnoses. Furthermore, vitamin D status was unknown at baseline and the dose of vitamin D supplementation varied across study participants. Therefore, it is possible that in patients receiving vitamin D supplementation, the plasma concentration of vitamin D was sub-optimal to cause an immunomodulatory effect. In future studies, vitamin D levels should be measured at baseline, making it possible to analyse the effects of vitamin D supplementation separately for vitamin D deficient and sufficient patients. However, it must be kept in mind that the serum 25-hydroxyvitamin D concentration needed for optimal immune function is unclear. The current recommendations for adequate 25-hydroxyvitamin D levels and vitamin D supplementation are based on amounts needed for skeletal health.[48]

The ultimate study design to definitively assess the effect of vitamin D supplementation on the risk of pneumonia would be an intervention study in a high risk population, such as nursing home residents. Study participants should be randomised to either sufficiently high doses of vitamin D or placebo. 25-hydroxyvitamin D measurements at baseline and during follow-up are

needed to assure adequate supplementation. At this moment, there is an ongoing study in Japan in institutionalised elderly investigating the prevention of pneumonia by vitamin D supplementation, with a follow-up time frame of 1 year (http://clinicaltrials.gov NCT 00877422).

Whether adjuvant vitamin D supplementation can play a therapeutic role in the acute care of patients with CAP also is currently unknown. In Chapter 9 of this thesis, we demonstrated that vitamin D deficiency at the time of hospital admission with CAP is associated with an increased risk of ICU admission and 30-day mortality. If this association is based on a causal relationship, vitamin D supplementation next to antibiotic therapy might be a promising new treatment option in CAP. Literature suggests that vitamin D can dampen the cytokine response.[37] Therefore, we propose to conduct a pilot study in patients with CAP, randomising them to either a high dose of vitamin D or placebo at the time of hospital admission. Serial measurement of pro- and anti-inflammatory cytokines during the course of disease should be performed. This pilot study may answer the question whether timing of the intervention at the moment of clinical expression of the disease would be a feasible approach.

Factors complicating the use of immunomodulatory agents in CAP

The use of immunomodulatory drugs in CAP patients is complicated by several factors. First, the immune response in CAP is very heterogeneous. Both the nature of the cytokine response (i.e. the relative contribution of individual cytokines) and the kinetics may vary between patients. In Chapter 2 of this thesis, we show that this can partly be explained by the microbial aetiology of CAP: different microorganisms trigger different profiles of inflammatory markers. This may indicate that the benefit of adjuvant immunomodulatory agents is variable among different pathogens. Indeed, in Chapter 4, downregulation of the cytokine response by dexamethasone was more pronounced in CAP caused by atypical pathogens than in pneumococcal pneumonia, and thus seems to be dependent on the causative microorganism. Thus, application of adjuvant therapy, which is fixed in timing, dose and duration, may be suboptimal or even counterproductive in some patients.

Another complicating factor is the appropriate timing of administration of immunomodulatory agents. From sepsis models, we are aware of a biphasic immune response in critically ill patients that varies over time.[49, 50] In the early phase of sepsis, the pro-inflammatory response predominates, while in the later phase the anti-inflammatory response becomes predominant (Figure 1).

It is unclear to what extent this sepsis model applies to CAP patients. Though, a

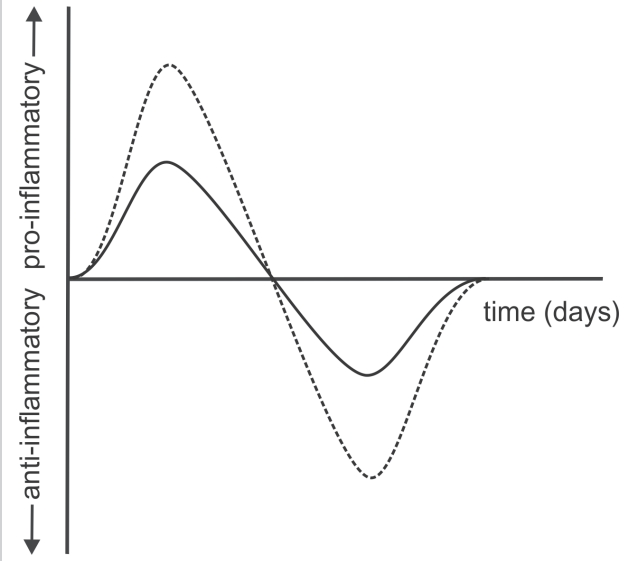

Figure 1. The biphasic inflammatory response in sepsis over time. During the early phase, the pro-inflammatory response predominates, while in the later phase the anti-inflammatory response prevails. The time span is unknown, and may vary from patient to patient. The intensity of the individual response may vary, due to virulence of the microbe, inoculum size, patient's comorbidities, polymorphisms in genes for cytokines etcetera.[49, 50]

vast number of patients with CAP fulfils the criteria of sepsis. Sepsis is defined as infection plus systemic manifestations of infection.[51] In our cohort, including patients with disease severity ranging from mild to severe, 84% of the patients met the sepsis criteria. The time point at which patients present to the hospital with CAP is known, but not the 'day zero', i.e. the moment of start of infection. From this, and from the fact that the time span of the biphasic response is variable, it is unclear at which point on the graph patients are admitted. Therefore, a measure of the 'degree of inflammation' should be developed to establish the phase of the inflammatory response, and hence, assist in guiding of administration of immunomodulatory agents. Hypothetically, in case of a hyper-immune state, dampening of the inflammatory response by immunomodulatory agents may be beneficial, while in a hypo-immune state this should be avoided. The latter conclusion leads to the next point of consideration: in clinical trials, patients with a known immunodeficiency or immunosuppressive medication are excluded from study participation. However, also milder forms of immune dysfunction

due to comorbidities such as diabetes mellitus and renal insufficiency should be taken into account, when considering treatment with immunomodulatory agents. Finally, the potential benefit of the immunomodulatory agents should always be balanced against potential negative side effects associated with the individual drugs.

Summarising conclusions and future perspectives

CAP is a common disease with considerable morbidity and mortality, despite effective antibiotic treatment. A promising new treatment option is modulation of the immune response. Macrolides, vitamin D and corticosteroids are all known to possess immunomodulatory properties and are therefore potential candidates for adjuvant treatment strategies in CAP.

For the near future, adjuvant treatment with corticosteroids seems most promising. In this thesis, we showed a net overall beneficial effect of dexamethasone treatment on length of hospital stay in non-immuno-compromised patients with CAP. In addition, our data indicate that certain subgroups of patients might benefit in particular from dexamethasone treatment. In CAP patients with a high pro-inflammatory cytokine response, but a discrepantly low cortisol, adjuvant dexamethasone treatment was associated with a significant decrease in mortality/ICU admission (combined endpoint). Future research should therefore not only focus on confirmation of the effectiveness of corticosteroids in the overall CAP population, but should also be directed towards identification of subgroups of patients who benefit most from corticosteroid therapy. Potential factors that might influence the effectiveness of corticosteroid therapy in CAP are the immune status of the patient, the microbial aetiology of CAP, the time point at which patients present at the hospital with CAP and the nature (and intensity) of the inflammatory response.

With respect to macrolides, the exact mechanisms of immunomodulation during acute inflammation have not been fully elucidated yet. Additional *in vitro* experiments are needed to conclude whether adjuvant macrolides have a favourable effect on the cytokine response during acute inflammation or not. The clinical benefit of adjuvant macrolides in CAP has not been proven unequivocally. To definitively assess whether adjuvant macrolide therapy is effective in CAP, large RCTs comparing β-lactam monotherapy with a combi-

nation of β-lactam antibiotics and macrolides are needed. The Netherlands is one of the few countries in which such a trial can be conducted, since CAP guidelines in many other countries already recommend standard combination therapy for all patients presenting with CAP. We are anxiously awaiting the results of the ongoing trials on this subject. When the value of macrolides as adjuvant therapy in CAP is established, a desirable next step would be to develop macrolide derivatives with immunomodulatory activity, but without antibacterial effects, in order to prevent an increase in macrolide-resistance.

The role of vitamin D supplementation in the prevention of pneumonia remains uncertain. Our data demonstrate no preventive effect of vitamin D supplementation on the risk of pneumonia in adults. This suggests that there is no causal relation between vitamin D deficiency and the risk of pneumonia. Vitamin D deficiency seems to be just a marker of frailty and poor prognosis. However, the absence of a preventive effect might also be due to shortcomings of our observational study. To ultimately asses the effect of vitamin D supplementation on the risk of pneumonia, an intervention study in a high risk population, such as nursing home residents, is needed.

Whether vitamin D supplementation in the acute care of CAP can improve disease outcome should be investigated in future studies. In this thesis, we demonstrate that vitamin D deficiency at the time of hospital admission with CAP is associated with an increased risk of ICU admission and 30-day mortality, and can act as a predictor of 30-day mortality. However, it is unclear whether the association between vitamin D deficiency and adverse outcome in CAP is based on a causal relationship. This, combined with uncertainty about the optimal dose for its immunomodulatory activities and timing of administration, vitamin D supplementation to patients with CAP is not recommended yet. Future studies should first clarify these uncertainties.

For immunomodulation in general, detailed characterisation of the cytokine response during the course of CAP can possibly provide a framework with a window of opportunity to intervene. The right immunomodulatory agent should be given to the right selection of patients, at the right dose, at the right time and for the right duration. These aspects should be further substantiated in future studies.

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Introductie

Longontsteking is een veel voorkomende ziekte, die wereldwijd verantwoordelijk is voor aanzienlijke morbiditeit en mortaliteit. Ook in Nederland komt longontsteking vaak voor, met een geschatte incidentie van 8,3 per 1.000 mannen per jaar, 8,4 per 1.000 vrouwen per jaar, en een nog hogere incidentie bij kinderen en ouderen. In 2010 werden 35.409 personen opgenomen in het ziekenhuis met een longontsteking en 5.413 personen overleden aan (de gevolgen van) een longontsteking.

Longontstekingen kunnen worden ingedeeld naar de plaats waar de longontsteking wordt opgelopen: 'buiten het ziekenhuis opgelopen' (community-acquired), 'in het ziekenhuis opgelopen' (hospital-acquired) of 'opgelopen aan de beademing' (ventilator-associated). Dit proefschrift richt zich op de buiten het ziekenhuis opgelopen longontsteking, in medische termen community-acquired pneumonia.

Een longontsteking is een infectie van het longweefsel, veroorzaakt door een micro-organisme, meestal een bacterie of een virus. De meest voorkomende bacteriële verwekker van buiten het ziekenhuis opgelopen longontsteking is *Streptococcus pneumoniae* (de pneumokok). Ook *Haemophilus influenzae* en de zogenaamde atypische bacteriën (*Legionella pneumophila, Mycoplasma pneumoniae, Chlamydophila* species en *Coxiella burnetii*) komen veel voor. Een longontsteking geeft veelal klachten van hoesten, opgeven van slijm, benauwdheid en pijn op de borst. De ontstekingsreactie in het lichaam zorgt voor symptomen zoals koorts of juist onder-temperatuur, transpireren en koude rillingen. Bloedonderzoek toont vaak verhoogde ontstekingswaarden. Op de longfoto wordt een ontsteking gezien.

De belangrijkste pijler in de behandeling van longontsteking is op dit moment het toedienen van antibiotica. Ondanks de beschikbaarheid van effectieve antibiotica is de sterfte ten gevolge van longontsteking de laatste decennia niet verder afgenomen. Om de uitkomst van longontsteking verder te verbeteren zijn daarom nieuwe, aanvullende behandelstrategieën nodig. In dit proefschrift worden opties voor aanvullende behandeling van buiten het ziekenhuis opgelopen longontsteking beschreven, door aan te grijpen op de afweerreactie van het lichaam (modulatie van de immuunrespons ofwel immuunmodulatie).

De immuunrespons tijdens longontsteking

Het menselijk lichaam kan zich op drie manieren verdedigen tegen binnendringende micro-organismen: via de mechanische afweer, via het

aangeboren immuunsysteem en via het verworven immuunsysteem. De mechanische afweer in de luchtwegen bestaat uit mechanische barrières en reflexmechanismen, zoals trilhaar(bewegingen), hoesten en niezen. Mocht een micro-organisme desondanks toch het lichaam binnendringen, dan stuit het in eerste instantie op het aangeboren immuunsysteem. Het aangeboren immuunsysteem bestaat uit witte bloedcellen die geactiveerd worden door ontstekingsstoffen (cytokines) en eiwitten die binden aan bacteriën zodat ze daarna makkelijker opgeruimd kunnen worden (complement). Het verworven immuunsysteem komt pas na enkele dagen tot weken op gang en wordt gekenmerkt door andere typen witte bloedcellen. Het aangeboren immuunsysteem werkt snel, maar is minder specifiek voor de ziekteverwekker. De verworven immuunreactie daarentegen kost tijd, maar is zeer specifiek voor het binnendringende micro-organisme en heeft geheugen.

Het binnendringen van een micro-organisme in de longen wekt een lokale ontstekingsreactie op. De aard van de ontstekingsreactie wordt bepaald door het type micro-organisme. De bestaande kennis over micro-organisme specifieke patronen van ontsteking is echter beperkt. Daarom hebben we in **hoofdstuk 2** een uitgebreid panel van ontstekingsstoffen (leukocyten getal, CRP, procalcitonine en 11 verschillende cytokines) gemeten in het bloed van 469 patiënten met een longontsteking. De ontstekingsreactie van patiënten met een longontsteking veroorzaakt door *Streptococcus pneumoniae* werd vergeleken met die van patiënten met een longontsteking door een atypische verwekker of een virus. Er wordt geconcludeerd dat de verschillende verwekkers van longontsteking een eigen profiel van ontstekingsstoffen opwekken in de gastheer. Antibiotica gebruik voor opname in het ziekenhuis gaf geen significante verandering van dit profiel.
Een ontstekingsreactie is essentieel voor de verdediging tegen binnendringende micro-organismen. Echter, een overmatige ontstekingsreactie is ongunstig en kan bijdragen aan een slechter beloop van de ziekte. Remming of aanpassing van de ontstekingsreactie (immuunmodulatie) lijkt daarom een veelbelovende behandelstrategie voor longontsteking. In dit proefschrift focussen we op de immuunmodulatoire eigenschappen van corticosteroïden (groep van geneesmiddelen die zijn afgeleid van bijnierschorshormonen), macroliden (een bepaald type antibiotica) en vitamine D, en hun potentiële rol in het voorkomen en/of behandelen van longontsteking.

Immuunmodulatie door dexamethason

Een van de potentiële behandelopties tijdens longontsteking is de toediening van corticosteroïden naast antibiotica. Corticosteroïden kunnen de ontstekingsreactie tijdens een longontsteking afremmen. Toediening van corticosteroïden is al effectief gebleken in een aantal andere infectieziekten, zoals hersenvliesontsteking veroorzaakt door bacteriën, of septische shock (ziektebeeld waarbij een bacterie in de bloedbaan aanleiding geeft tot prikkeling van de vaatwand, waardoor deze open gaat staan, wat leidt tot verlaagde bloeddruk en versnelde pols). Echter, de effectiviteit van corticosteroïden in buiten het ziekenhuis opgelopen longontsteking is nog niet onomstotelijk bewezen: voorgaande studies toonden zeer wisselende resultaten. Daarom hebben we een grote studie uitgevoerd naar het effect van het toevoegen van dexamethason aan antibiotica op de opnameduur in het ziekenhuis tijdens een longontsteking. Deze studie wordt beschreven in **hoofdstuk 3**. Volwassen patiënten met een longontsteking werden willekeurig toegewezen aan de testgroep, die werd behandeld met dexamethason (een bepaald type corticosteroïd) of aan de controlegroep, die werd behandeld met placebo (een middel dat geen werkzame bestanddelen bevat). Het was een zogenaamd dubbelblind onderzoek, waarbij noch de patiënt noch de behandelend arts wist of de patiënt dexamethason of placebo toegediend kreeg. Patiënten bekend met een niet functionerend of onvoldoende functionerend immuunsysteem werden uitgesloten van de studie. De testgroep kreeg gedurende 4 dagen 5 mg dexamethason toegediend, eenmaal per dag. De placebogroep kreeg steriel water toegediend op dezelfde momenten. In totaal deden 304 patiënten mee aan het onderzoek, waarvan 151 patiënten dexamethason kregen en 153 placebo. Vergeleken met placebo gaf dexamethason 1 dag verkorting van de opnameduur. Daarnaast lieten patiënten in de dexamethasongroep een snellere daling van bepaalde cytokines zien dan patiënten in de placebogroep. Een bekende bijwerking van behandeling met dexamethason is het ontstaan van te hoge bloedsuikers (hyperglycemie). Hyperglycemie werd vaker gerapporteerd in de dexamethasongroep, maar ernstige bijwerkingen waren zeldzaam. De frequentie van ernstige bijwerkingen verschilde niet tussen beide groepen.

De invloed van dexamethason op de cytokine respons tijdens longontsteking wordt verder onderzocht in **hoofdstuk 4**. In het bloed van de voornoemde 304 patiënten is een uitgebreid panel van cytokines gemeten. Vergeleken met placebo-behandelde patiënten, toonden dexamethason-behandelde patiënten een snellere daling van de cytokines. Dit effect was verschillend

voor de diverse microbiële verwekkers van longontsteking. Bij patiënten met longontsteking door *Streptococcus pneumoniae* had dexamethason weinig invloed op de cytokine concentraties, terwijl bij patiënten met longontsteking door een atypische verwekker dexamethason juist een snellere daling van bepaalde cytokine concentraties werd gezien.

Het gunstige effect van dexamethason toediening suggereert dat tijdens een longontsteking de lichaamseigen regelmechanismen van ontsteking onvoldoende werken. Cortisol is het lichaamseigen corticosteroïd dat door de bijnierschors wordt aangemaakt. Cortisol is een belangrijk ontstekingsremmend hormoon. Normaal gesproken neemt de productie van cortisol door de bijnierschors in het lichaam toe tijdens ernstige ziekte en stress. Een te lage cortisol productie door de bijnierschors bij ernstig zieke patiënten is geassocieerd met een slechte ziekte uitkomst. In de literatuur wordt gesuggereerd dat deze patiënten baat hebben bij toediening van corticosteroïden in medicijnvorm. In het onderzoek beschreven in **hoofdstuk 5** werd onderzocht of het mogelijk is om op basis van de cortisol concentratie tijdens longontsteking subgroepen te definiëren die in het bijzonder baat hebben bij corticosteroïd therapie. Dit onderzoek toonde aan dat patiënten met hoge cytokine waarden, maar daarbij een onevenredig laag cortisol, met name baat hadden bij dexamethason therapie. Namelijk, binnen de groep met hoge cytokine waarden en een onevenredig laag cortisol, hadden de dexamethason-behandelde patiënten geen IC opname nodig en overleed niemand, terwijl dit wel het geval was bij 43% van de placebo-behandelde patiënten. Op basis van deze uitkomsten kan worden gesteld dat correlatie van cortisol aan de cytokine respons mogelijk het beste aangeeft welke patiënten het meeste baat hebben bij corticosteroïd therapie.

Naast het feit dat een te lage cortisol productie geassocieerd is met een slechte ziekte uitkomst, staat in de bestaande literatuur ook beschreven dat een hoge cortisol concentratie bij ziekenhuisopname met longontsteking geassocieerd is met een slechte uitkomst. Of aanhoudende hoge cortisol spiegels gedurende de opname met longontsteking ook geassocieerd zijn met een slechte ziekte uitkomst is onbekend. Dit is onderzocht in **hoofdstuk 6**, waarin een reeks van cortisol metingen tijdens longontsteking wordt beschreven. Aanhoudende hoge cortisol spiegels bleken inderdaad geassocieerd te zijn met een slechte ziekte uitkomst. Een potentieel risico van behandeling met corticosteroïden is onderdrukking van de lichaamseigen productie van bijnierschorshormonen. De tijd die het lichaam nodig heeft om de eigen

productie van bijnierschorshormonen te herstellen na korte behandeling met dexamethason tijdens infectie is onbekend. De data in **hoofdstuk 6** laten zien dat de lichaamseigen cortisol productie na een eerste gift dexamethason bijna volledig onderdrukt wordt. Op dag 30 na opname is de lichaamseigen cortisol productie weer volledig hersteld.

Immuunmodulatie door macroliden

Het is bekend dat macrolide antibiotica immuunmodulatoire eigenschappen bezitten naast hun directe effect op bacteriën. De immuunmodulatoire eigenschappen van macroliden zijn bewezen effectief in chronische ontstekingsziekten van de long, zoals diffuse panbronchiolitis, cystic fibrosis, astma en bronchiëctasieën. In hoeverre macroliden ook immuunmodulatoire effecten hebben tijdens acute ontsteking, zoals bij longontsteking, is minder duidelijk.

In **hoofdstuk 7** geven we een overzicht van het bewijs in de bestaande literatuur voor immuunmodulatoire effecten van macroliden tijdens longontsteking. Macroliden blijken de aard van de immuunrespons tijdens acute ontsteking op 3 manieren te veranderen: door onderdrukking van de cytokine respons, door verandering van het gedrag van ontstekingscellen en door beïnvloeding van structurele cellen van de luchtwegen. De huidige bewijsvoering wordt echter beperkt door de verschillen tussen de studies met betrekking tot methoden en de gebruikte experimentele modellen. Daarnaast ontbreken studies naar het immuunmodulatoire effect van macroliden wanneer deze worden toegevoegd aan een veelgebruikt antibioticum tijdens longontsteking, namelijk β-lactam antibiotica.

Om de mechanismen van immuunmodulatie door macroliden verder te ontrafelen, in het bijzonder wanneer gegeven in combinatie met β-lactam antibiotica, hebben we een reageerbuis model van acute infectie door *Streptococcus pneumoniae* ontworpen. In **hoofdstuk 8** laten we zien dat macroliden, alleen of gecombineerd met β-lactam antibiotica, de cytokine respons dempen in bloed gestimuleerd met hitte-gedode pneumokokken. Dit suggereert een immuunmodulatoir effect van macroliden. Dit effect werd niet waargenomen in experimenten met levende pneumokokken. Stimulatie van bloed met levende pneumokokken veroorzaakte een overweldigende cytokine respons, maar er werd geen verschil in cytokine respons waargenomen tussen behandeling met een combinatie van macrolide en β-lactam antibiotica, en behandeling met alleen β-lactam antibiotica.

Immuunmodulatie door vitamine D

Vitamine D heeft, naast zijn bekende functie in de calcium- en bothomeostase, ook immuunmodulatoire eigenschappen. Vitamine D tekort komt wereldwijd veel voor en is geassocieerd met een verhoogd risico op luchtweginfecties. Bij patiënten opgenomen in verband met longontsteking is vitamine D tekort geassocieerd met een slechter klinisch beloop. De voorspellende waarde van de vitamine D concentratie tijdens longontsteking voor het beloop van ziekte is echter onbekend. **Hoofdstuk 9** laat zien dat 53% van onze studiepatiënten met longontsteking een vitamine D tekort had. Onze data bevestigen dat een tekort aan vitamine D geassocieerd is met een slechter ziekte beloop. Patiënten met een vitamine D tekort gingen vaker dood binnen 30 dagen na opname in het ziekenhuis dan patiënten zonder vitamine D tekort. Patiënten met een vitamine D tekort werden vaker opgenomen op de intensive care (IC) dan patiënten zonder vitamine D tekort. De vitamine D concentratie op het moment van ziekenhuisopname (ingedeeld in 3 categorieën) bleek een goede voorspeller te zijn voor dood binnen 30 dagen na opname (30-dagen mortaliteit). Als de vitamine D status bij opname werd toegevoegd aan de Pneumonia Severity Index (PSI) score (veelvuldig gebruikt hulpmiddel in de dagelijkse praktijk om de het risico op dood binnen 30 dagen na opname met longontsteking vast te stellen) nam de voorspellende waarde voor 30-dagen mortaliteit significant toe.

De vraag is of er ook een oorzakelijke relatie is tussen vitamine D tekort en verhoogd infectierisico, en tussen vitamine D tekort en een slechte ziekte uitkomst. Is dit het geval, dan zou het toedienen van vitamine D aan mensen met een hoog risico op het krijgen van een longontsteking een preventief effect hebben, en zou het toedienen van vitamine D aan patiënten met een longontsteking het klinisch beloop gunstig kunnen beïnvloeden.

De invloed van vitamine D suppletie op het risico van het krijgen van een longontsteking wordt beschreven in **hoofdstuk 10**. Drie grote, onafhankelijke studies toonden aan dat vitamine D suppletie niet geassocieerd was met een lager risico op het ontwikkelen van een longontsteking. In één van de studies werd zelfs een toegenomen risico op longontsteking gevonden bij vitamine D gebruikers. In dit hoofdstuk wordt gesteld dat op basis van deze bevindingen, (standaard) vitamine D suppletie niet kan worden aanbevolen ter voorkoming van longontsteking.

Conclusies en aanbevelingen

De studies in dit proefschrift geven meer inzicht in de mogelijke toepassing van corticosteroïden, macroliden en vitamine D als behandeling naast antibiotica tijdens longontsteking, of ter voorkoming van longontsteking. Veel vragen blijven echter nog onbeantwoord. Toekomstig onderzoek dient het gevonden positieve effect van corticosteroïden op de opnameduur in het ziekenhuis bij een longontsteking te herbevestigen. Verder onderzoek zal zich ook moeten richten op de identificatie van subgroepen van patiënten die het meeste baat hebben bij corticosteroïd therapie. Ten aanzien van macroliden, dient toekomstig onderzoek meer helderheid te verschaffen over de precieze mechanismen van immuunmodulatie tijdens acute infectie en zijn verdere studies nodig om de klinische effectiviteit van macrolide therapie naast standaard antibiotica tijdens longontsteking vast te stellen. Dit proefschrift laat zien dat de vitamine D concentratie ten tijde van ziekenhuisopname met longontsteking een goede voorspeller is van de 30-dagen mortaliteit. Echter, de rol van vitamine D toediening in de behandeling van longontsteking blijft vooralsnog onduidelijk. Op basis van de bevindingen in dit proefschrift kan vitamine D suppletie ter voorkoming van longontsteking niet worden aanbevolen.

Over immuunmodulatie in het algemeen tijdens longontsteking stellen we dat het juiste immuunmodulatoire medicijn moet worden gegeven aan de juiste selectie van patiënten, in de juiste dosis, op het juiste moment en voor de juiste tijsduur. Deze aspecten van immuunmodulatie moeten verder worden uitgewerkt in toekomstig onderzoek.

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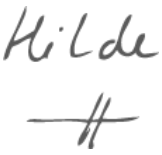

Curriculum vitae

Hilde Hilligje Femmigje Remmelts werd geboren op 16 april 1984 in Kampen. Na het behalen van haar gymnasium diploma aan het Marnix College te Ede, startte zij met de studie Geneeskunde aan de Universiteit Utrecht. In het laatste jaar van haar studie maakte ze reeds kennis met wetenschappelijk onderzoek op de afdeling Interne geneeskunde en Infectieziekten van het UMC Utrecht (Dr. P. Ellerbroek). Na het behalen van haar arts-examen in 2008, begon zij als 'arts-assistent niet in opleiding' op de afdeling Interne Geneeskunde van Ziekenhuis Gelderse Vallei in Ede. Een half jaar later werd Hilde aangenomen voor de opleiding tot internist, in respectievelijk ziekenhuis Gelderse Vallei te Ede (opleider: Dr. R. Heijligenberg) en het Universitair Medisch Centrum Utrecht (opleider: destijds Prof. dr. D.H. Biesma, thans Prof. dr. M.M.E. Schneider). Tijdens haar opleiding in Ede werd Hilde gevraagd de coördinatie van de Ovidius studie op zich te nemen: een studie naar het effect van dexamethason op de opnameduur van patiënten met longontsteking. Deze studie was opgezet in het St. Antonius Ziekenhuis te Nieuwegein (promovenda Sabine Meijvis, promotoren Prof. dr. D.H. Biesma en Prof. dr. J.C. Grutters), met Ede als 2^{e} deelnemende ziekenhuis. Vele patiënt inclusies later werd Hilde door Prof. dr. D.H. Biesma gevraagd of zij interesse had om een deel van de data van de Ovidius studie te gebruiken voor een eigen promotie-traject. Een concreet onderzoeksplan werd opgesteld, vanuit een samenwerkingsverband tussen 3 ziekenhuizen (ziekenhuis Gelderse Vallei te Ede, St. Antonius ziekenhuis te Nieuwegein en Universitair Medisch Centrum Utrecht). Op 1 januari 2011 onderbrak Hilde officieel haar opleiding tot internist voor dit promotie-onderzoek. Hilde woont samen met haar vriend Jeroen Verbocht in Lunteren. Zij zijn sinds 3 september 2012 de trotse ouders van Wiesje.