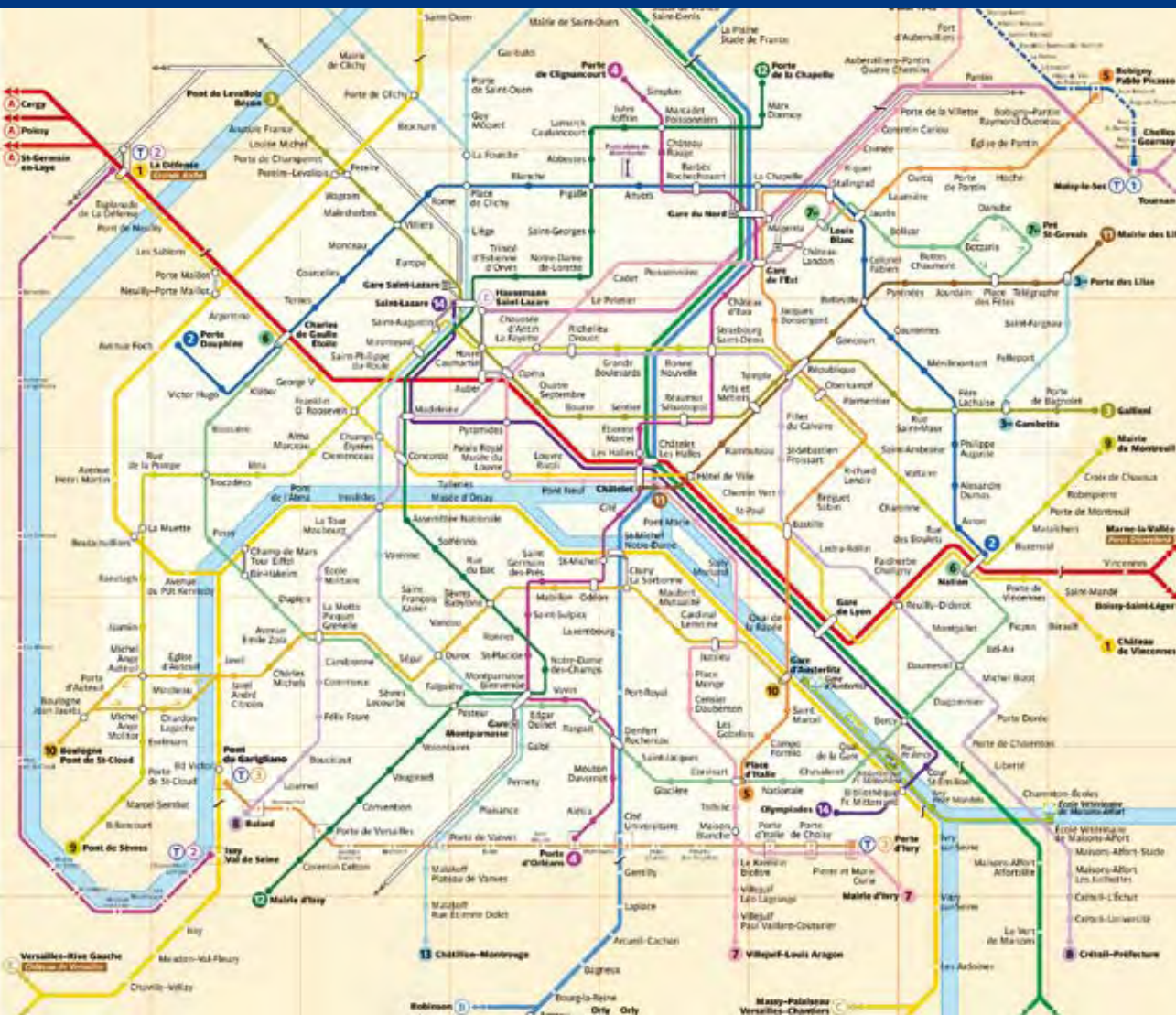
Clinical characteristics and innate immunity in patients with community-acquired pneumonia



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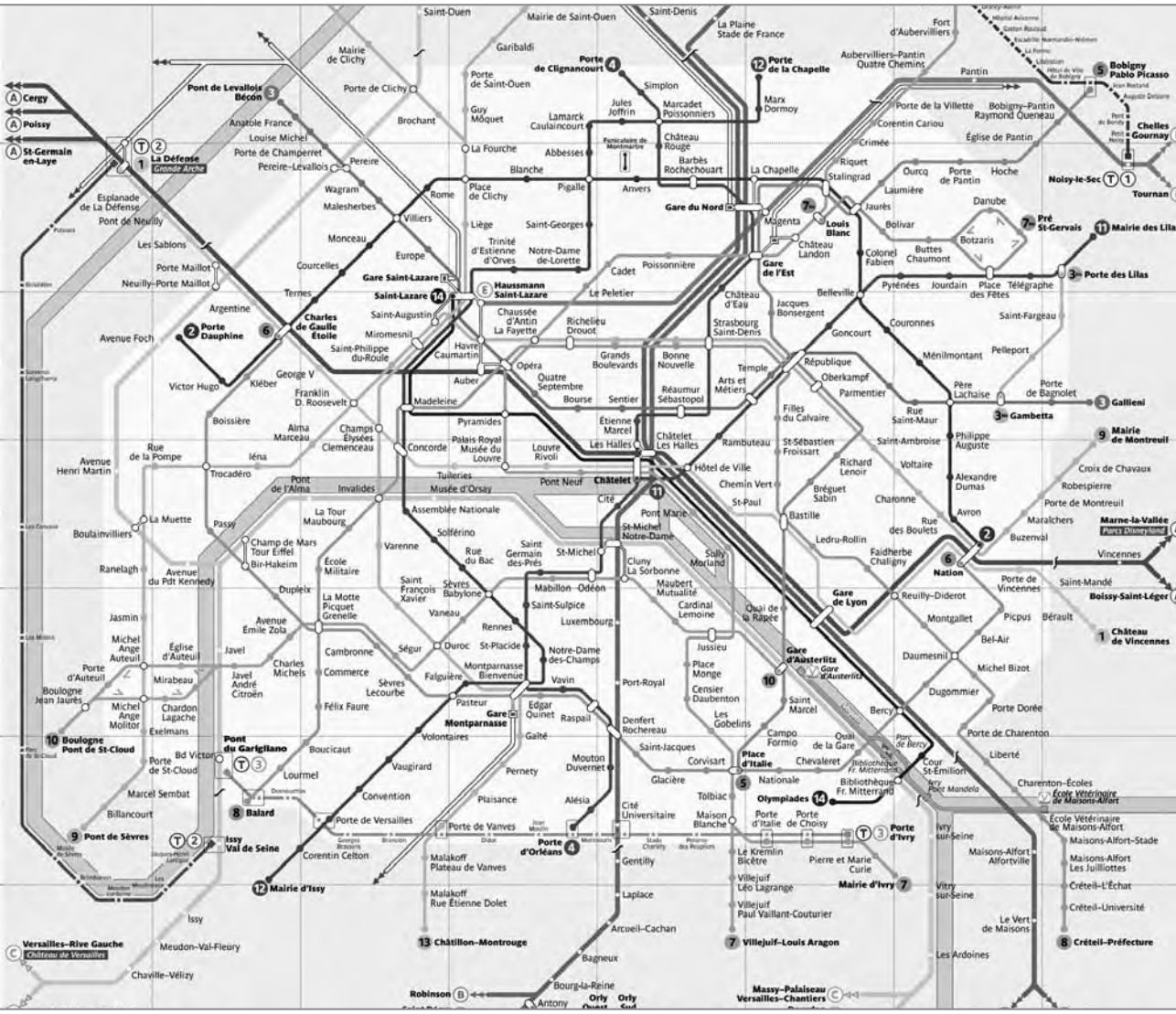

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Klinische kenmerken en het humorale afweersysteem van patiënten met een extramuraal ontstane longontsteking
(met een samenvatting in het Nederlands)

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	Dr. G.P. Voorn

Contents

General introduction and aim of the thesis

Introduction

Community-acquired pneumonia (CAP) is the most common infectious disease requiring hospitalisation in the Western world. In spite of improving antibiotic regiments, CAP still has significant mortality [1-4]. In non-immune compromised patients, *Streptococcus pneumoniae* (pneumococcus) is the most frequently isolated micro-organism in CAP, followed by the Gram-negative bacterium *Haemophilus influenzae* [2, 4-11]. There are many known risk factors for CAP caused by a specific pathogen [1, 4, 5]. Nevertheless, many patients, especially young patients, do not contain any of these risk factors for CAP. A possible explanation could be that these patients are at risk for CAP caused by a specific micro-organism due to genetic variations within their immune system. The outline of this thesis is to identify such variations within a part of the immune system: innate immunity.
In the first part of this thesis, the results of an extensive diagnostic protocol using different diagnostic tools in patients with CAP will be described. This section will contain the results of this procedure, including the causative micro-organisms of CAP, efficiency of the used diagnostic tools and patient characteristics. A proposal for a diagnostic protocol will be presented. This part will be followed by an intermezzo, in which the interaction between ACE polymorphisms, susceptibility to pneumonia and ACE serum levels will be described. The second part of this thesis will cover the genetic make-up of the innate immune system in relation to susceptibility to and the clinical course of CAP, with a focus on cytokine responses, receptors in the innate immune system (Toll-like receptors and the Fcγ receptor IIa), and the mannose-binding lectin pathway.

Part I: Clinical characteristics

Causative micro-organisms

Streptococcus pneumoniae is the most frequent causative micro-organism of CAP, followed by *Haemophilus influenzae*. *Mycoplasma pneumoniae* or *Legionella pneumophila* is generally considered as the third most frequently identified causative micro-organism in patients with CAP [2, 4-11]. These two bacteria together with *Chlamydophila pneumoniae*/*psittaci* and *Coxiella burnetii* form the group of 'atypical' bacteria due to their lack of the classical signs of pneumonia [12-16]. *Staphylococcus aureus* is another frequently identified Gram-positive bacterium, and *Klebsiella pneumoniae* is the second most common non-atypical Gram-negative bacterium. CAP can also be caused by respiratory viruses [2, 4-11, 17, 18].

Diagnostic tools

The number of diagnostic tools used to identify the causative micro-organisms of CAP is still growing. Classical diagnostic tools are culturing of sputum or blood, but sensitivity is low, and definite results take time [19]. There are urine antigen tests available for *Streptococcus pneumonia* and *Legionella pneumophila*, both with high specificity, and results are quickly available [20-30]. Nevertheless, sensitivity for both tests is moderate to low, and the added value of the pneumococcal

urine antigen test is unknown. Polymerase chain reaction (PCR) with sputum samples can help to identify *Chlamydophila pneumoniae*/*psittaci*, *Legionella pneumophila* and *Mycoplasma pneumoniae* [31-33]. The place of PCR within the diagnostic work-up for CAP is still unclear. Viruses are cultured in sputum or in samples taken by pharyngeal swab [17-18]. Respiratory viruses and (atypical) bacteria can also be detected serologically, but definite results need paired sampling and are therefore not directly available [12, 13, 15, 16, 19, 34, 35]. Without positive pathogen identification, it is impossible to perform pathogen-guided antibiotic therapy, which is advised in most international guidelines, though most of them do not contain a diagnostic protocol [36-42].

Patient characteristics

In the past, clinical characteristics were used to identify patients with atypical pneumonia [12-16]. For example, frequently observed findings in CAP caused by *Legionella pneumophila* were high fever, high inflammatory parameters, hyponatremia and increased serum levels of hepatic enzymes and creatininekinase [14]. The added value of the use of this so-called 'syndromic approach' is a matter of debate, as is the use of the term 'atypical pneumonia' [12, 13, 16]. Most protocols for CAP contain the Legionella urine antigen test and anti-Legionella antibiotics [36-42]. The results of this test are available very quickly, making identification of atypical pneumonia caused by *Legionella pneumophila* by clinical characteristics unnecessary. Furthermore, the specificity and especially the sensitivity of these clinical characteristics are far too low to use them for microbial diagnosis in CAP [12-14, 16]. There are, however, no fast diagnostics for *Mycoplasma pneumoniae*, *Chlamydophila* species and *Coxiella burnetii*. The clinical characteristics of patients with CAP caused by any of these micro-organisms are less well described and they cannot be distinguished from other causative micro-organisms of CAP. Atypical pneumonia is mainly diagnosed in young patients without co-morbidities [12-16]. COPD is a risk for CAP caused by *Haemophilus influenzae* and high age and cardio-pulmonary co-morbidities are associated with pneumococcal pneumonia [1, 4].

Intermezzo: ACE polymorphisms

As ACE-inhibitors, ACE polymorphisms resulting in lower ACE serum levels are reported to be protective against CAP [43-46]. Possible explanations for this finding are improved clearance of contaminated secretions in the upper airways, prevention of aspiration or immune modification by decreasing levels of angiotensinogen II [47-54]. Till so far, these findings were limited to Asian populations, and whether this association is also present in non-Asian populations is unknown [55].

The ACE I/D polymorphism determines the levels of ACE in serum [56]. ACE serum levels have been reported to quickly decrease in the acute phase of diseases such as ARDS and CAP, although with large individual differences [57-59]. The effect of the ACE polymorphism on this decrease, as its clinical relevance, is unknown.

Part II: Innate immunity

Cytokine response

The cytokine response in CAP can be divided into the pro-inflammatory cytokine response, with interleukin-6 (IL-6) as most important cytokine, and the anti-inflammatory cytokine response, most prominently reflected by interleukin-10 (IL-10). Both inflammatory responses are present in patients intrapulmonary and in the systemic circulation at the same time [60-63].

IL-6 is a pro-inflammatory acute phase protein of the innate immune system. Its function consists of delayed apoptosis and enhanced cytotoxicity of neutrophils, T-cell proliferation, development of antigen-specific T-lymphocytes, B-cell stimulation and activation of coagulation pathways [64-67]. IL-6 also contains anti-inflammatory effects including inhibition of the pro-inflammatory cytokines TNFα and IL-1 [68, 69]. In mice, IL-6 is essential for elimination of bacteria from the pulmonary compartment, and in its absence (knock out mice models), mortality from pneumococcal airway infection is higher [70, 71]. *Streptococcus pneumoniae* and its lipopolysaccharide (LPS) stimulate IL-6 production in vitro and in vivo [72-76]. IL-6 levels are primarily elevated in the lung, but they are also elevated in the systemic circulation in human bacterial pneumonia and sepsis [60-63]. IL-6 serum levels are associated with severity of disease [70, 72, 75, 78-86]. Within the IL-6, the IL-6 -174 C/T polymorphism, which is associated with level of IL-6 serum levels, is reported to be associated with infectious diseases [87-93].

IL-10 is an anti-inflammatory cytokine and plays an important role in the innate immune response against viral and bacterial pathogens. IL-10 is necessary to counterbalance pro-inflammatory cytokines, however, an excess of IL-10 is able to induce immunosuppression in sepsis and increases morbidity and mortality in pneumococcal pneumonia [94-96]. High concentrations of IL-10 on admission are associated with non-survivors of sepsis in general and severe sepsis due to CAP [97-98]. Individual variation between individuals in the magnitude of the IL-10 response during CAP was recently shown to be dependent on genetic background [99]. Single nucleotide polymorphisms (SNPs) have been associated with different IL-10 levels [100-102]; for example, the IL-10 -1082 G genotype is associated with increased IL-10 release and seems to be a risk factor for septic shock in pneumococcal infection [103]. The relation between IL-10 polymorphisms and CAP in general is unknown.

Toll-like receptors and the Fcγ receptor IIa

Toll-like receptors (TLRs) are transmembrane receptors and activate an inflammatory response upon recognising pathogen-associated molecular patterns. TLR2 is able to bind bacterial lipoproteins and lipoteichoic acids, TLR4 binds LPS, and TLR5 acts as a flagellin receptor [104-110]. The role of TLRs in pneumococcal diseases and pneumonia has been examined in experimental models. TLR2$^{-/-}$ mice infected intracerebrally with *Streptococcus pneumoniae* had a more disseminated disease and reduced survival as compared to wild-type controls. A comparable TLR2$^{-/-}$ model of pneumococcal pneumonia, however, showed no differences in outcome between wild-type and knock-out mice [10-12]. Pneumococcal pneumonia in TLR4$^{-/-}$ mice was characterised by a slightly reduced inflammatory response. On the other hand, TLR4$^{-/-}$ mice with Gram-negative (including Legionella) pneumonia had significantly lower bacterial clearance and survival as compared to wild-type mice [13-18]. The role of TLR5 has not been studied in a mouse model of pneumonia. Functional polymorphisms are described in the genes of all three TLR, but the role of these polymorphisms in pathogen-specific CAP is largely unknown.

The only receptor able to interact efficiently with IgG2 antibodies is the Fcγ receptor IIa (CD32). This receptor is expressed on a variety of cells in the immune system, including lymphocytes, macrophages, and polymorphonuclear leukocytes (PMN). Binding of immune complexes by the Fcγ receptor IIa (Fcγ-RIIa) of PMNs can induce degranulation and phagocytosis [120, 121]. Both *Streptococcus pneumoniae* and *Haemophilus influenzae* are surrounded by a polysaccharide capsule that protects the bacteria from phagocytosis. If IgG2 subclass antibodies recognise and bind capsular polysaccharide antigens, opsonophagocytosis can take place [122-124]. Due to a single nucleotide polymorphism, two co-dominant Fcγ-RIIa allotypes exist with different binding capacities for IgG2 (R131 and H131). Fcγ-RIIa-H131 has a high affinity for IgG2, whereas Fcγ-RIIa-R131 displays a low affinity for IgG2 [120-121]. As a result, phagocytosis of IgG2-opsonised bacteria by homozygous Fcγ-RIIa-R/R131 PMNs is less efficient than phagocytosis by homozygous Fcγ-IIa-H/H131 PMNs [125]. The role of this polymorphism in pathogen-specific CAP is unknown.

Mannose-binding lectin pathway

Mannose (or mannan) binding lectin (MBL) is a calcium-dependent collagenous serum lectin produced by the liver during the acute phase response of inflammation. MBL binds to carbohydrates (mannose or N-acetylglucosamine) on the surface of different micro-organisms such as influenza A virus, pneumococci, *Haemophilus influenzae* and *Legionella pneumophila*; therefore, it is called a pattern-recognition molecule [126-128]. After binding, MBL mediates complement activation and opsonophagocytosis by activation of different mechanisms, including the MBL pathway of the complement system [129-134]. Within the MBL2 gene, both promoter and structural polymorphisms are known, and they are organised into six haplotypes. The effect of MBL polymorphisms on MBL serum levels is a matter of debate, and results are conflicting. In general, MBL haplotypes code for normal or deficient MBL serum levels [135-139]. MBL deficiency is associated with an increased general risk of severe infection in immune compromised patients [140-144]. In other patient groups, MBL deficiency is associated with risk of bacteremia, sepsis and fatal outcome, and the recurrence of respiratory infections and infections with capsulated bacteria [145-152]. The role of MBL deficiency in CAP in general is unknown and the role of MBL deficiency in pneumococcal invasive disease unclear.

Outline of the thesis

The central theme of this thesis is to identify variations within the innate immune system associated with susceptibility to and the outcome of CAP. In experimental setting, the effect of genetic diversity in the immune system is restricted to pneumonia caused by a specific micro-organism; therefore, the causative micro-organisms of CAP need to be identified. There are patient characteristics which are also risk factors for CAP caused by a specific pathogen, like COPD for CAP caused by *Haemophilus influenzae* and young age for atypical pneumonia. These risk factors for CAP should be explored in order to analyse the additive risk of genetic variations in innate immunity.
Causative micro-organisms of CAP, including the diagnostic tools to identify them, and patient characteristics will be described in part I. The role of genetic variations in innate immunity will be described in part II. The intermezzo will contain the results of the effect of the ACE I/D polymorphism in susceptibility to, the outcome of, and ACE serum levels in, CAP.

The central questions in part I will be: What are the causative micro-organisms of CAP, and which patient characteristics are linked to these micro-organisms? In order to identify the causative micro-organisms, different diagnostic tools can be used, including bacterial and viral cultures, urine antigen testing, PCR of sputum samples for atypical bacteria, and paired serological sampling. The results of these tests will be evaluated in Chapter 1, and diagnostic protocol for microbial diagnosis will be presented. In many patients, no causative micro-organism will be identified.
In order to prevent unnecessary expensive diagnostics, and more importantly, to identify patients in whom pathogen-guided antibiotic therapy is impossible, the characteristics of patients with negative pathogen identification will be evaluated (Chapter 2). A common denominator for negative pathogen identification is prior antibiotic treatment, and whether this also influences the bacterial aetiology of CAP will be answered in Chapter 3.
CAP caused by *Legionella pneumophila* and *Mycoplasma pneumonia* is called atypical pneumonia due to the non-pulmonary complaints of these patients and the absence of clear radiological features on chest X-ray. The term 'syndromic approach' means that attempts are made to identify the causative micro-organism of CAP by clinical characteristics thought to be specific for atypical pneumonia (especially Legionnaires' disease). In Chapter 4, the patient and clinical characteristics of the main four causative micro-organisms of CAP, in addition to atypical bacteria, will be described. The usefulness of the syndromic approach will also be discussed. Furthermore, this chapter will identify patient characteristics associated with the risk for CAP caused by specific pathogens.

In the intermezzo, the central question which will be answered is whether the ACE I/D polymorphisms is associated with susceptibility to and the clinical outcome of CAP (Chapter 5).
Chapter 6 will show the course of ACE serum levels during CAP, corrected for the ACE I/D polymorphism, and discuss its clinical relevance.

The central question in the part II of this thesis will be of whether variations in innate immunity are associated with susceptibility to and the clinical outcome of CAP. This thesis is restricted to three parts of innate immunity: the cytokine response, TLRs and the Fcγ-RIIa and the MBL-pathway.
The role in susceptibility to CAP of genetic variations in genes of two cytokines involved in the general inflammatory response, will be described in Chapters 7 (IL-6) and 8 (IL-10). Besides susceptibility, the association of these polymorphisms and serum levels of both interleukins with clinical outcome will be evaluated.
The effects of functional polymorphisms within the TLR2, TLR4 and TLR5 genes on susceptibility to pathogen-specific CAP will be discussed in Chapter 9. The question of whether variations in these three pathogen-recognition receptors affect the outcome of CAP is described, together with their effects on the inflammatory response as measured by C-reactive protein and IL-6. The results of a similar analysis will be reported for the Fcγ-RIIa in Chapter 10.
The question of whether MBL2-genotypes are associated with pathogen-specific CAP will be described in Chapter 12, including their effects on clinical outcome. Before that, however, the relationship between MBL2-genotypes, MBL-haplotypes and the level of circulating MBL will be described (Chapter 11). This chapter will also answer the question of whether MBL is an acute phase protein or not.
This thesis concludes with a summary of the results and a general discussion.

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Part I: Clinical characteristics

Chapter 1

A three-step diagnostic model for identification of causative micro-organisms in community-acquired pneumonia

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Abstract

Background

In this study, we introduce a diagnostic model for identification of causative micro-organisms in patients with community-acquired pneumonia.

Methods

In 201 consecutive patients, we performed cultures of sputum and blood, pneumococcal and Legionella urine antigen tests, polymerase chain reaction (PCR) for atypical bacteria, viral culture of the pharynx and serologic testing for viruses and atypical bacteria. Based on the test results, we constructed a diagnostic model.

Results

A three step diagnostic model was constructed. Culture of sputum and blood, and urine antigen testing in the first step revealed all non-atypical bacteria, and 29% of atypical bacteria. By adding the PCR in the second step, 76% of the atypical bacteria were identified. The third step, containing viral culture and paired serological testing, had no consequences for the treatment of the patients.

Conclusion

We present a tree-step diagnostic model, in which in the first two steps all clinical relevant pathogens were identified.

Introduction

Community-acquired pneumonia (CAP) is the most common infectious disease requiring hospitalization in the Western world [1-3]. CAP is caused by numerous micro-organisms. *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Staphylococcus aureus* can be identified by positive culture of these bacteria in sputum or, in the case of the Gram-positive bacteria, blood [4]. For the detection of *Streptococcus pneumonia*, a pneumococcal urine antigen test is available [5]. Identification of atypical bacteria is more difficult. A urine antigen test is available for screening of *Legionella pneumophila* [6]. Polymerase chain reactions (PCRs) of sputum can help to identify *Chlamydophila pneumoniae* and *psittaci*, *Legionella pneumophila* and *Mycoplasma pneumoniae* [7]. Viruses are cultured in sputum or samples or in samples taken by pharyngeal swab or can be detected serologically [4, 8-10].

Early detection of the causative micro-organism of CAP makes pathogen-guided antibiotic therapy possible; this is advocated in most international guidelines [11-15]. By constructing a diagnostic model, aimed at maximal pathogen identification, one could increase the number of patients treated following the pathogen-guided concept, and at the same time limit unnecessary testing.

This study describes a population of patients with CAP. An extensive diagnostic protocol was used to identify the causative micro-organism of CAP. Aim of this study was to design a step-wise diagnostic model for micro-organism identification in CAP based on the outcome of available diagnostic tests and used in our population in the detection of the causative micro-organism.

Methods

Patients

All patients (>18 years) with CAP presenting in the period October 2004 - August 2006 in a general 600-bed teaching hospital in the centre of the Netherlands were included in this study. Patients with a history of recent hospitalization (<30 days) or a congenital or acquired immunodeficiency (including those treated with prednisone 20 mg per day, or equivalent, for more than three days) were excluded. A pneumonia was defined as a new infiltrate on the chest X-ray and two out of six clinical signs of pneumonia (cough, production of sputum, signs of consolidation on respiratory auscultation, temperature >38°C or <35°C degrees Celsius, white blood count (WBC)>$10x10^9$/l) or WBC<$4x10^9$/l) or more than 10% rods in the differentiation, and C-reactive protein (CRP) 3 times above the upper limit of normal (>15 mg/l). The chest X-ray was interpreted by a resident in the emergency department. Within 24 hour after presentation, the chest X-ray was evaluated by an experienced radiologist, who was not aware of the clinical course of the patient. The following data were collected at presentation: age, sex, medical history and Fine-score [16]. This study was approved by the local Medical Ethics Committee and informed consent was obtained from all patients.

Pathogen identification

The diagnostic protocol for pathogen identification consisted of cultures of sputum and blood, urine antigen testing for *Streptococcus pneumoniae* and *Legionella pneumophila*, polymerase chain reac-

tion (PCR) of the sputum for atypical bacteria, serologic testing for atypical bacteria and respiratory viruses and viral culture of the pharynx. At the moment of presentation in the emergency department, two samples of peripheral blood were taken for culture. Blood cultures were regarded as being positive in the case of positive culturing of a respiratory pathogen. Next to blood, sputum cultures were taken at presentation, or as soon as possible within the first 24h of hospitalization. Gram-stain was made of all samples and judged by experienced microbiological laboratory assistant. Sputum was regarded as non-representative if Gram-stain showed more than 50 epithelial cells per microscopic view (10x magnification) or if the number of epithelial cells is between 25-50 cells per view in absence of leukocytes. When more than one micro-organism was found, the micro-organism with the most abundant growth was considered as the causative micro-organism. Urine samples were taken for antigen testing on *Streptococcus pneumoniae* (Binax *S. pneumoniae* kit) and *Legionella pneumophila* (Binax Legionella urine antigen test) [5, 6]. Urine was sampled at admission in the emergency department or, in case of initial anuria, within the first 24 hours of hospitalization. Polymerase chain reactions (in house developed Taqman real-time PCR) were performed of sputum in order to detect DNA of *Chlamydophila pneumoniae* and *psittaci*, *Legionella pneumophila* and *Mycoplasma pneumoniae* [5]. The (preliminary) results of cultures and urine antigen testing were available within 24 hours. The results of the PCRs of the sputum were available within the next 24 hours (within 48 hours after presentation). Sampling for serologic testing on the presence of antibodies to *Coxiella burnetii*, *Mycoplasma pneumoniae*, influenza-virus A and B, parainfluenza-virus, 1, 2 and 3, adenovirus and respiratory syncytial virus was performed on day 1 and 10 of hospitalization (Complement fixation CBR) [10]. Serologic sampling for *Legionella pneumophila* was done on day 1 and 30 (IgG/IgM virion/serion ELISA Clindia) [17]. Only samples with a four fold rise in antibody titres were considered positive. Pharyngeal samples were taken for viral culture. Viral pneumonia was defined by positive viral testing (culture or seroconversion) in combination with the presence of negative cultures and antigen tests for bacterial micro-organisms. Mixed infections were defined as mixed bacterial infections (positive culture of sputum and/or blood, positive urine antigen test, positive PCR or positive serological results) and as mixed bacterial-viral infection (positive bacterial testing in combination with positive viral testing). In case of mixed bacterial testing, the micro-organism identified in blood was considered as the causative micro-organism of CAP, followed by a positive urine antigen test, positive PCR, positive serological results and positive culture of sputum. In case of mixed bacterial-viral infection, the bacterium was considered as the causative micro-organism of CAP.

Statistical analysis

We used the SPSS 12.0 package for statistical analysis. Descriptive statistics are given as mean or median, standard deviation (SD) or range.

Results

Initially 255 patients were included in this study. In 201 cases a new infiltrate on chest X-ray was confirmed by an experienced radiologist. Demographics of these 201 patients are shown in Table 1. Forty-eight patients (24%) had received antibiotics before admission (self-reportage). Most frequently used antibiotics were amoxicillin-clavulanate (n=16; 33% of all patients using antibiotics), amoxicillin (10, 21%) and clarithromycin (9, 19%). Ten patients (5%) died during hospitalization (all Fine class V), 21 (10%) needed treatment in the ICU-department. Median duration of hospital stay was 11 days, ranging from 3 up to 153 days.

Table 1: Demographics of 201 patients with community-acquired pneumonia

Characteristic		
Male sex, n (%)		124 (62)
Age, mean (SD)		63 (17)
COPD, n (%)		61 (30)
Pre-hospital antibiotic therapy, n (%)		48 (24)
Fine-class	I, n (%)	30 (15)
	II, n (%)	34 (17)
	III, n (%)	53 (26)
	IV, n (%)	56 (28)
	V, n (%)	28 (14)

In 128 patients (64%) the causative micro-organism of CAP was identified. As shown in Table 2, *Streptococcus pneumoniae* was the most frequently identified pathogen (n=60; 30%), followed by *Haemophilus influenzae* (14; 7%), *Legionella pneumophila* (9; 4%) and *Mycoplasma pneumoniae* (9; 4%). Most frequently identified causative micro-organisms in patients with COPD *were Streptococcus pneumoniae* (35%) and *Haemophilus influenzae* 14%), and in patients with pre-hospital antibiotic treatment *Streptococcus pneumoniae* (17%) and *Mycoplasma pneumoniae* (8%). *Streptococcus pneumoniae* was the most frequently identified causative micro-organism of CAP in all Fine-classes, followed by atypical bacteria in Fine-classes I, II and III, and by *Haemophilus influenzae* in Fine-class IV and V. Mixed infections were found in 24 (12%) patients, of which 17 patients with combined bacterial-viral infections (mainly Streptococcus pneumoniae in combination with a respiratory virus). In 7 patients combined bacterial infections were found.

Table 2: Causative micro-organisms in 201 patients with community-acquired pneumonia

Micro-organism	Number (%)
Streptococcus pneumoniae	60 (30)
Haemophilus influenzae	14 (7)
Legionella pneumophila	9 (4)
Mycoplasma pneumonia	9 (4)
Staphylococcus aureus	6 (3)
Influenza-virus	5 (2)
Klebsiella pneumoniae	4 (2)
Other viruses[1]	11 (5)
Other non-atypical Gram-negative bacteria[2]	5 (2)
Other atypical bacteria[3]	3 (1)
Other Gram-positive bacteria[4]	2 (1)
Unknown	73 (36)

1 adenovirus, herpes simplex virus, para-influenza-virus and respiratory syncytial virus

2 *Acinetobacter calcoaceticus*, *Escheridia coli*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*

3 *Chlamydophila pneumoniae*/*psittaci* and *Coxiella burnetii*

4 non-pneumococcal streptococci

Pneumococcal pneumonia (n=60) was diagnosed by culture of *Streptococcus pneumonia* in sputum (n=22, 37%), positive urine antigen test (15; 25%), positive blood culture (5; 8%) or a combination of these tests (18; 30%). In 20 out of 33 (61%) cases of culture of *Streptococcus pneumoniae* in sputum and in 6 out of 17 (35%) in blood the urine antigen test remained negative. The urine antigen test was the only positive diagnostic test in 15 (25%) patients with pneumococcal pneumonia. *Haemophilus influenzae* pneumonia was in all cases diagnosed by positive culture of sputum. *Legionella pneumophila* urine-antigen test was positive in 6 out of 9 (66%) patients with Legionnaires' diseases. PCR for *Legionella pneumophila* of sputum was positive in all 5 available samples. All cases with a positive PCR had a positive urine antigen test. Serology was positive in all 7 (100%) available paired samples (in the remaining 2 cases no paired samples available), including 3 patients with a negative Legionell*a* urine antigen test. *Mycoplasma pneumoniae* was identified as the causative pathogen in 9 patients: 7 patients had a positive PCR (in 5 patients serological confirmed) and 2 patients had positive serology only (in one patient negative PCR and in one patient no available sputum). The remaining non-atypical gram-stained negative and positive bacteria were identified by culture of sputum, the atypical bacteria by PCR (*Chlamydophila* species) or serology (*Coxiella burnetii*), and the viruses by serology and/or culture.

Table 3: Diagnostic techniques in 201 patients with community-acquired pneumonia

Diagnostic technique	Tested	
	Total (%)	*Positive (%)*
Culture of sputum	148 (74)	69 (47)
Culture of blood	182 (91)	19 (10)
Urinary antigen test for *Legionella pneumophila*	186 (93)	6 (3)
Urinary anitgen test for *Streptococcus pneumoniae*	183 (91)	30 (16)
PCR of sputum for atypical bcateria[1]	78 (39)	14 (18)
Viral culture of the pharynx[2]	88 (44)	14 (16)
Serology for *Coxiella burnetii* and *Mycoplasma pneumoniae*[3]	161 (80)	9 (6)
Serology for respiratiory viruses[4]	161 (80)	22 (14)
Serology for *Legionella pneumophila*[5]	130 (65)	7 (5)

1 *Chlamydophila pneumoniae* and *psittaci*, *Legionella pneumophila* and *Mycoplasma pneumoniae*
2 Influenza viruses, para-influenza viruses and HSV1
3 paired sample on day 1 and day 10
4 paired sample on day 1 and day 10 for adenovirus, influenza viruses, para-influenza viruses and respiratory syncytial virus
5 paired sample on day 1 and day 30

The results of the used diagnostic techniques used for pathogen identification are shown in Table 3. We succeeded to collect material for culture of blood and urine antigen testing in more than 90% of the patients. In all cases of positive *Streptococcus pneumoniae* or *Legionella pneumophila* urine antigen testing, no other bacterial causative pathogens were identified. Collection of sputum was successful in 74% of all patients; the remaining patients were unable to produce representative sputum. PCR of the sputum was available in a part of the sputum samples (53%), but its efficiency was, next to direct culture of the sputum, high (18% positive result). Serologic testing for respiratory viruses, *Mycoplasma pneumoniae* and *Coxiella burnetii* was performed in 80% and for *Legionella pneumophila* in 65% of the patients. Paired sampling was not available in case of discharge or death before day 10 of hospitalization, death before control visit or lost to follow-up.
On basis of our findings, we developed a three step model to identify the causative pathogen of CAP (Figure 1). The first step contains culture of sputum and blood and urine antigen testing for *Streptococcus pneumoniae* and *Legionella pneumophila*. In case of negative testing after 24h, PCR of the sputum for atypical bacteria is added in step 2. If testing remains negative, and in case of clinical interest, viral culture of the pharynx can be performed and serologic diagnostics are added in step 3 to identify all other causative micro-organisms.

In the first step, a microbial diagnosis would be made in 97 out of 201 patients (48%) within 24 hours after admission. All non-atypical bacteria would have been identified by conventional cultures of sputum and blood in combination with the *Streptococcus pneumoniae* urine antigen test, and 6 out of 9 patients with *Legionella pneumophila*. In case of positive testing for Legionella, an antibiotic against *Legionella pneumophila* could be added to the therapy, which is standard of care in our

hospital. Similar counts for changing initial broad-spectrum antibiotics to penicillin or amoxicillin in the patients with positive pneumococcal testing (sputum and/or urine antigen test). No further testing would be necessary in these 97 patients. In our population, antibiotic change was made in 2/3 of the patients with positive urine antigen testing.

After 24 hours of negative testing in step 1, the remaining 104 patients would have entered step 2. In this step, the causative micro-organism of another 9 (5%) patients would have been identified. The nature of these pathogens, all *Mycoplasma pneumoniae* or *Chlamydophila* species, would result in antibiotic switch, which was done in all our 9 patients. The results of the PCR were available within 48 hours after admission.

In the remaining 95 patients no pathogen would have been identified within the first 48h. In case of special interest, one could perform step 3 to gain final identification of the causative micro-organism of CAP, but the results of these tests need time (due to paired sampling or culture). In our population, this would identify the causative micro-organism in another 22 patients (11%). This group of patients consists of 15 patients with viral pneumonia, which has no therapeutic consequences, and 7 patients with atypical pneumonia with a benign course: 3 Legionella urine-antigen test negative patients with Legionella pneumonia, 2 patients with *Mycoplasma pneumonia* and one with Q-fever. All serological diagnosis was made after discharge from hospital, thereby limiting clinical consequences.

Discussion

We identified a causative micro-organism in 64% of patients with CAP. *Streptococcus pneumoniae* (30%), *Haemophilus influenzae* (7%), *Legionella pneumophila* (4%) and *Mycoplasma pneumoniae* (4%) were the most frequent identified causative micro-organisms. This is in accordance with previous reports [1-3, 18-21]. Positive identification of the causative pathogen makes micro-organism guided antibiotic therapy possible, which was done in the majority of patients in our group. Micro-organism guided antibiotic therapy is advised in most guidelines on the treatment of CAP, but they do not contain diagnostic protocols [11-15]. We constructed a simple three-step diagnostic protocol, which would have identified all clinical relevant pathogens in our population and could be tested in prognostic studies.

The first step of the three-step diagnostic protocol consists of cultures of sputum and blood in combination with pneumococcal and Legionella urine antigen testing. This step is performed within the first 24 hour after admission. The aim of this step is to identify all non-atypical bacteria and *Legionella pneumophila*. The latter due to its detrimental clinical course and need for specific antibiotic therapy [22-24]. In this step, all non-atypical Gram-negative bacteria were identified by positive cultures of sputum. By combining sputum cultures with cultures of blood and *Streptococcus pneumoniae* urine antigen testing, all Gram-positive bacteria were identified. After this step, 48% of patients had a microbial diagnosis, leading to adjustment of antibiotic therapy. Further evaluation of the causative micro-organism in this group of patients would not have been necessary, as there were no clinical relevant mixed bacterial infections. In the second step, PCR of the sputum is performed in search for atypical bacteria. This step is initiated after negative pathogen-identification in step 1 and completed

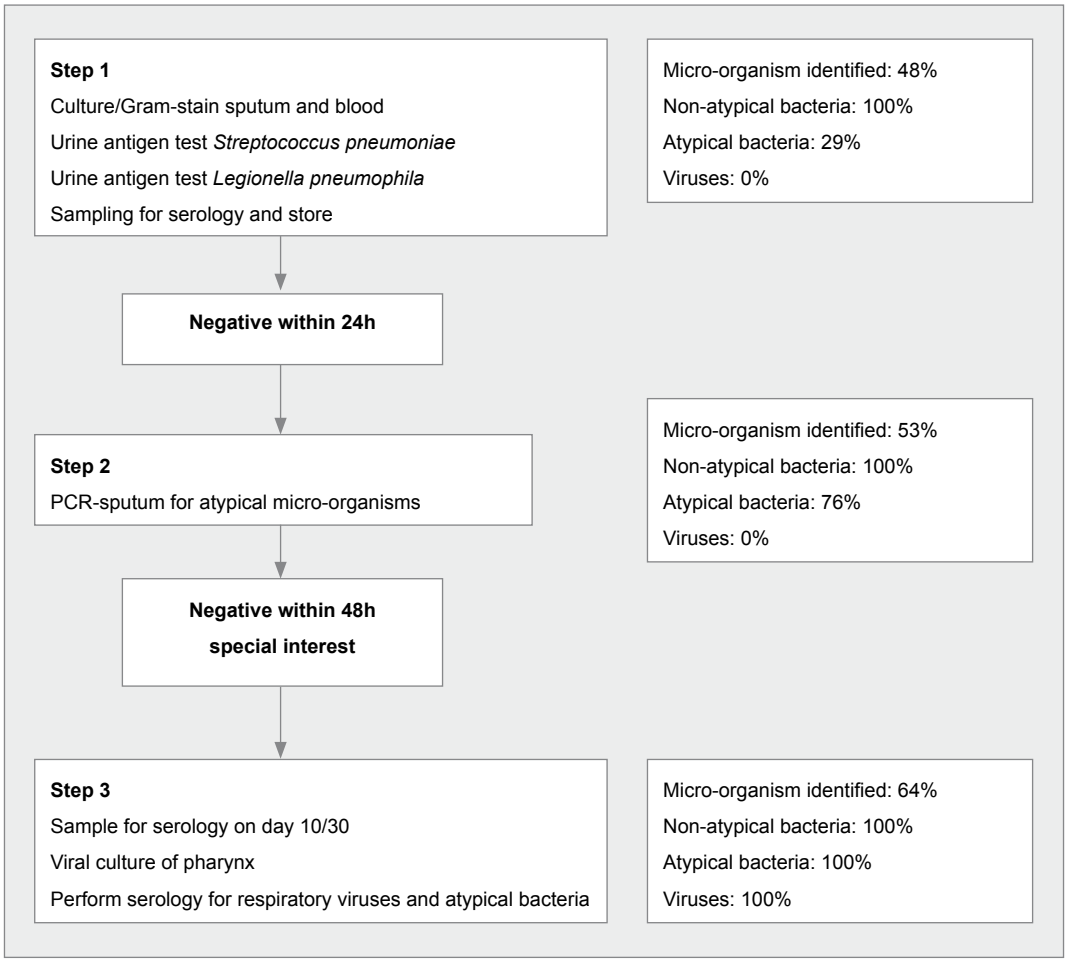
48 hours after admission. Aim of this step is to identify atypical bacteria, which need specific antibiotic therapy. In another 5% of the patients with CAP is microbial diagnosis is made, which resulted in an antibiotic switch in all patients. The third diagnostic step identifies the remaining 11% of identified causative pathogens of CAP. In this step viral cultures of the pharynx are combined with serologic testing for atypical bacteria and respiratory viruses. The aim of this step is to monitor the frequency of causative micro-organisms of CAP, but has no direct therapeutic consequences. A definite serological diagnosis is made after paired sampling with an interval of 10 days; in many cases microbial diagnosis would have been made after discharge form hospital.

In step 1, the *Streptococcus pneumoniae* positive urine antigen test was in some cases the only positive finding, but was also found to be negative in the presence of positive culture of sputum (61%) and/or blood (35%); so it sensitivity is limited. The sensitivity of this test is a matter of debate [25-31]. Negative urine antigen testing in combination with positive cultures of sputum and/or blood was not associated with pre-hospital antibiotic therapy. Next to the pneumococcal urine antigen test, the Legionella urine antigen test is performed in step 1. This is a sensitive and specific test to identify *Legionella pneumophila* serotype 1 [4, 31]. Other serotypes can be identified by using PCR or serologic techniques, which explains our finding of 3 patients with negative urine antigen test in combination with positive PCR or serologic results. In step 2, *Mycoplasma pneumoniae*, *Chlamydophila* species were identified by using PCR techniques of the sputum. The usefulness of PCR for detection of these atypical bacteria has been reported before [22, 32-33].

This study was constructed to develop a diagnostic protocol, which could be tested in a prognostic cohort study. Its sample size is too small to draw definite conclusions, especially because some of the used tests were not performed in all patients. Not a limitation, but complementary with its design, is the debate whether the identified pathogen is the true causative micro-organism of CAP, especially in patients with viral pneumonia, or patients with a history of COPD and Gram-negative pneumonia diagnosed on basis of positive culturing of sputum. Another limitation is that, though most guidelines advice pathogen-directed antibiotic therapy, the effect on clinical outcome of this strategy is a matter of debate [34, 35].

In conclusion, we introduce a three-step diagnostic protocol for pathogen identification in patients with CAP. We succeeded in positive pathogen-identification in 64% of the patients. The first step consists of cultures of sputum and blood and urine antigen testing. After 24 hour, in 48% of patients a pathogen is identified. The second step consists of PCR of the sputum 48 hours after admission and will add another 5% positive pathogen-identification, all atypical bacteria. The remaining 11% is identified in step 3, mainly viral pneumonia. The first and second step are important steps to make pathogen-guided antibiotic treatment possible, the third step can be performed for epidemiological interest. This diagnostic model should be tested in prospective studies to evaluate cost-efficiency and number of patients needed to screen.

Figure 1: Three-step diagnostic protocol for community acquired pneumonia and percentage of micro-organisms identified by step in 201 patients



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Part I: Clinical characteristics

Chapter 2

Clinical features predicting failure of pathogen identification in patients with community-acquired pneumonia

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Abstract

Background

Community-acquired pneumonia (CAP) is caused by a variety of micro-organisms. By identifying patients at risk for failure of pathogen identification, it is possible to make an early decision on the extent of diagnostic procedures to be performed. This is especially important in patients with severe CAP. The aim of this study was to identify these patients by using clinical and laboratory features.

Methods

In 201 patients hospitalized for CAP clinical and laboratory variables were collected. Pathogen identification was performed by culture of sputum and blood, urine antigen tests, polymerase chain reactions of sputum, serologic testing and viral culture of the pharynx.

Results

In 128 patients a respiratory micro-organism was identified. In both univariate and multivariate analysis failure of pathogen identification was predicted by pre-hospital antibiotic therapy, a medical history of hypertension and a low C-reactive protein.

Conclusion

We conclude that patients with pre-hospital antibiotic therapy, a medical history of hypertension and a relatively low C-reactive protein are at risk for failure of pathogen identification. These predictors should be confirmed in a larger population. Invasive testing in high-risk patients with CAP in the presence of these predictors should be considered in an early phase of hospitalization.

Introduction

Community-acquired pneumonia (CAP) is the leading cause of hospitalization for an infectious disease in the Western world [1]. CAP is most frequently caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila*, *Mycoplasma pneumoniae* and respiratory viruses [2-6]. Antibiotic therapy of these micro-organisms is different. In order to perform micro-organism guided therapy, it is necessary to identify the causative micro-organism of CAP. Many non-invasive diagnostic tools are available for pathogen identification: bacterial and viral culture of sputum, culture of blood, urine antigen testing for *Legionella pneumophila* (serotype 1) and *Streptococcus pneumoniae*, and polymerase chain reactions (PCR) to detect bacterial fragments of *Chlamydophila pneumoniae* and *psittaci, Mycoplasma pneumoniae* and *Legionella pneumophila* in respiratory samples. Serologic testing can be performed to detect antibodies to respiratory viruses and some atypical micro-organisms (including *Legionella pneumophila*) [7].

Most international guidelines advise to adjust the antibiotic regimen in case of positive micro-organism identification [8-11]. Successful implementation of micro-organism guided antibiotic therapy will need predictors of diagnostic failure of non-invasive diagnostic tools, thereby selecting those patients who would be eligible for more invasive diagnostic manoeuvres such as bronchoalveolar lavage. This is especially important in patients with severe CAP. In patients with the combination of low-risk pneumonia and high risk for negative pathogen identification, one could decide to withhold expensive or time-consuming non-invasive testing.

The aim of this study was to identify predictors for diagnostic failure of pathogen identification of standard non-invasive tests in patients hospitalized for CAP. Therefore, we compared clinical characteristics of patients with positive pathogen identification in CAP, with the characteristics of patients with negative testing. Positive pathogen identification was defined as positive testing of at least one of the non-invasive diagnostic tests (positive culture of sputum or blood, positive PCR of sputum, positive urine antigen test, serologic conversion in paired samples or positive viral culture) with a respiratory pathogen and the absence of positive testing in one of the other tests (except for viruses).

Methods

Patients

All patients (> 18 years) with CAP hospitalized in the period October 2004 - August 2006 in a general 600-bed teaching hospital were included in this study. Patients with a history of recent hospitalization (< 30 days) or a congenital or acquired immunodeficiency (including patients treated with prednisone 20 mg per day for more than three days) were excluded.

A pneumonia was defined as a new infiltrate on the chest X-ray and two out of six clinical signs of pneumonia (cough, production of sputum, signs of consolidation on respiratory auscultation, temperature >38 or <35 degrees Celsius, leukocytosis (WBC >10 G/l) or leukopenia (WBC <4 G/l) or more than 10% rods in the differentiatial count and C-reactive protein (CRP) 3 times above the upper limit of normal (5 mg/l). The chest X-ray was interpreted by the medical doctor in the emergency

department. Within 24 hour after presentation, the chest X-ray was evaluated by an experienced radiologist, who was not aware of the clinical course of the patient.

The following data were collected at presentation: age, sex, medical history, cough, sputum production, sounds at auscultation, blood pressure, heart rate, respiratory rate, temperature, pulse oximetry, Glasgow Coma Score, haemoglobin, white blood cell count (WBC), platelets, C-reactive protein (CRP), sodium, potassium, urea, creatinine, bilirubin, albumin, glucose and arterial blood gas. Antibiotics used at presentation were recorded as mentioned by the patients or their relatives, together with the duration of antibiotic therapy. Medical history was retrieved form the hospital medical record, or, if unavailable, from the medical record of the general practitioner. The following co-morbidities were reported: congestive heart failure (CHF), coronary heart disease, dysrythmias, myocardial infarction, heart valve failure, COPD, asthma, interstitial lung disease, CAP, repetitive upper airway and urinary infections, meningitis, malignancy (local, metastatic, haematological), diabetes mellitus (type I and II), thoracic surgery (CABG, valve replacement, pulmonary surgery), renal and hepatic failure, hypercholesterolemia, CVA, peripheral vascular disease, hypertension and thrombo-embolic diseases. This study was approved by the local Medical Ethics Committee and informed consent was obtained from each patient.

Pathogen identification

Of all patients sputum (if available) and blood (at least two samples) were cultured. All sputum samples were cultured; sputum samples with positive cultures were only used for further analysis if they fulfilled our definitions of representative sputum. Sputum was considered representative if less than 25 epithelial cells per view were present in the absence of leucocytes or if less than 50 epithelial cells per view were present if leukocytes were present. When more than one micro-organism was found, the micro-organism with the most abundant growth was considered as the causative micro-organism. Polymerase chain reactions (Taqman real-time PCR) were performed in sputum in order to detect DNA of micro-organisms causing atypical pneumonia (*Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydophila pneumoniae* and *psittaci)* [12]. Urine samples were taken for antigen testing on *Streptococcus pneumonia* (Binax *S. pneumoniae* kit) and *Legionella pneumophila* serogroup 1 (Binax Legionella urine antigen test) [13-14]. Sampling for serologic testing on the presence of antibodies to *Mycoplasma pneumoniae*, *Coxiella burnetii* or respiratory viruses (adenovirus, influenza virus A and B, para-influenza-viruses 1, 2 and 3, adenovirus and respiratory syncytial virus) was done at day 1 and 10 (Complement fixation), for *Legionella pneumophila* on day 1 and 30 (IgG/IgM virion/serion ELISA Clindia) [15]. Only samples with a clear seroconversion (a four fold rise in antibody titres) were considered positive. Pharyngeal samples were taken for viral culture (influenza viruses and herpes simplex virus type 1). Viral pneumonia was defined by positive viral testing (culture or seroconversion) in combination with the presence of negative cultures and antigen tests for bacterial micro-organisms.

Statistical analysis

Statistical analysis was done by using the SPSS 12.0 package. Differences between patients with and without known causative micro-organisms were calculated by Mann-Whitney tests, independent sample t-tests, one-way ANOVA or Chi-square exact tests. Logistic regression analysis (backward stepwise method) was performed containing the univariate significantly predicting variables of diag-

nostic failure of pathogen identification. To calculate the power of the predictive model found after logistic regression analysis a ROC curve analysis was performed. A difference $p < 0.05$ is considered as statistically significant, unless otherwise indicated.

Results

Initially 255 patients were included in this study. In 201 patients a new infiltrate on the X-ray was confirmed by an experienced radiologist and these patients were found eligible for this study (124 males and 77 females). Baseline characteristics are shown in Table 1. Reported co-morbidities were COPD (30%), hypertension (19%), diabetes mellitus (17%), peripheral vascular disease (11%), CHF (9%) and cerebro-vascular accident (8%). Three patients lived in a nursery home. Fine-classes IV and V consisted of 84 patients (42%), class III of 53 patients (26%) and class I and II of 64 patients (32%). In-hospital mortality was 5% (n = 10) and 21 (10%) patients were admitted to the ICU. Most of these patients were in Fine-class IV or V. Mortality in Fine-class IV/V was 11% versus 1% for mortality in patients with Fine-class I-III ($p = 0.002$). Similar was found for admission to ICU: 18% versus 5% ($p = 0.005$). Duration of hospitalization was also dependent of the Fine-class (one-way ANOVA; $p = 0.004$).

In total 148 sputum samples were collected, of which 69 (47%) cultures were positive and representative. Of the culture negative sputum samples, 78 were worked up for PCR, of which 14 (18%) were positive for *Mycoplasma pneumoniae*, *Legionella pneumophila* or *Chlamydophila* species. Nine-teen (10%) out of 182 blood samples taken for culture were positive. Urine antigen test for *Legionella pneumophila* and *Streptococcus pneumoniae* were positive in respectively 6/186 (3%) and 30/183 (16%) cases. Paired serologic samples for viruses were available in 161 cases, of which 22 (14%) were positive for respiratory viruses and 9 (6%) for *Coxiella burnetii* or *Mycoplasma pneumoniae*. Paired serology for *Legionella pneumophila* was available in 130 patients, of which 7 (5%) were positive. The number of tests in patients with positive and negative pathogen identification is nearly equal: mean 6.7 (SD 1.7) tests versus 6.3 (SD 1.8) tests respectively.

Table 1: Demographic characteristics of 201 patients with CAP

Characteristic		Micro-organism	
		Unknown (n = 73)	*Known* (n = 128)
Male Sex, n (%)		44 (60)	80 (63)
Age, mean (range)		66 (32-98)	62 (18-91)
CHF[1], n (%)		5 (7)	14 (11)
COPD, n (%)		19 (26)	44 (34)
CVA, n (%)		6 (8)	11 (9)
Diabetes, n (%)		13 (18)	22 (17)
Hypertension, n (%)		21 (29)*	17 (13)*
PVD[2], n (%)		10 (14)	12 (9)
Living in nursery, n (%)		2 (3)	1 (1)
Fine-class	I, n (%)	8 (11)	22 (17)
	II, n (%)	11 (15)	23 (18)
	III, n (%)	27 (37)	26 (20)
	IV, n (%)	19 (26)	37 (29)
	V, n (%)	8 (11)	20 (16)

1 CHF = congestive heart failure

2 PVD = peripheral vascular disease

* p = 0.007 Chi-square test

In 128 patients (64%) the causative micro-organism of CAP was identified. *Streptococcus pneumoniae* was identified as the causative micro-organism in 60 patients (30%), *Haemophilus influenzae* in 14 patients (7%), respiratory viruses in 16 patients (8%), *Mycoplasma pneumoniae* and *Legionella pneumophila* in 9 patients (4%) each and *Staphylococcus aureus* in 6 patients (3%). Other identified micro-organisms were (hemolytic) streptococci (n = 2), *Klebsiella pneumoniae* (n = 4), *Pseudomonas aeruginosa* (n = 2), *Chlamydophila* species (n = 2) and *Coxiella burnetii* (n = 1). Clinical course did not differ significantly between the different micro-organisms.

Table 2 shows variables predicting failure to identify the causative micro-organism of CAP. These variables consist of patient characteristics (medical history), laboratory characteristics (C-reactive protein) and characteristics of antibiotic treatment at the moment of presentation in the emergency department. Results of both univariate and multivariate analysis are shown. Pre-hospital antibiotic treatment (OR 2.20, CI 1.13-4.27, p = 0.019), a medical history of hypertension (OR 2.64, CI 1.28-5.41, p = 0.007), a low CRP (mean 184 mg/l (SD 120) versus 234 mg/l (SD 150), p = 0.035) and duration of antibiotic treatment before presentation (mean 1.3 days (SD 3.0) versus 0.5 days (SD 1.4), p = 0.006) predict failure to identify the causative micro-organism of CAP in univariate analysis. C-reactive protein and duration of pre-hospital antibiotic treatment were categorized in two groups. A CRP < 300 mg/l was a significant predictor for failure of pathogen identification (OR 3.99 (CI 1.82-8.75); p = 0.000). A non-significantly relation was found between duration of pre-hospital

antibiotic treatment (> 5 days) and failure of pathogen identification (OR 5.62 (CI 1.10-28.57); ns). No differences were found for sex, age, medical history (CHF, COPD, CVA, DM, peripheral vascular disease, living in nursery), Fine-class, duration of symptoms, clinical characteristics at presentation (blood pressure, heart frequency, temperature, respiratory frequency) and laboratory parameters (Hb, WBC, platelets, electrolytes, hepatic function, arterial blood gases and glucose). Multivariate analysis was performed with variables predicting failure of pathogen identification in univariate analysis. Antibiotic treatment at the moment of presentation (OR 2.71, CI 1.27-5.78, p = 0.01), a medical history of hypertension (OR 2.59, CI 1.21-5.50, p = 0.033) and a level of C-reactive protein < 300 mg/l (OR 3.45, CI 1.53-7.75, p = 0.003) predicted failure of pathogen identification in a multivariate model. The power of this model was calculated using ROC curve analysis: area under the curve was 0.60 (0.52-0.69; p = 0.016).

Table 2: Clinical characteristics predicting failure to identify causative micro-organism

Parameter	Micro-organism		Chi-square test	Logistic regression
	Unknown (n = 73)	*Known* (n = 128)	*OR (CI)*	*OR (CI)*
Antibiotic therapy[1], n (%)	24 (34)	24 (19)	2.20 (1.13-4.27)*	2.71 (1.27-5.78)*
Hypertension[2], n (%)	21 (29)	17 (13)	2.64 (1.28-5.41)*	2.59 (1.21-5.00)*
C-reactive protein > 300 mg/l	9 (12)	46 (36)	3.99 (1.82-8.75)*	3.45 (CI 1.53-7.75)*
Antibiotic therapy > 5 days[3]	6/68[4] (9)	2/118[4] (2)	5.62 (1.10-28.57)	3.73 (0.59-23.5)

1 Number of patients (%) with antibiotic therapy at the moment of presentation

2 Number of patients (%) with a medical history of hypertension

3 Duration of antibiotic therapy before presentation > 5 days

4 In some patients antibiotic therapy at the moment of presentation was unknown

* p< 0.05

Table 3a shows the relation between identification of positive pathogen identification in CAP and clinical outcome. As shown, no differences were found for in-hospital mortality, number of patients admitted to the ICU and length of hospitalization. Table 3b shows the effects of positive pathogen testing in CAP and antibiotic treatment. Many differences are found. In general, antibiotic therapy is more often switched in patients with positive pathogen identification (48%) compared to patients with unknown causative micro-organism (40%), although this difference does not reach the level of statistical significance. Antibiotic switching in patients with known micro-organisms is mainly done in the first three days of hospitalization (46/60 (76%), compared to 17/29 (59%) in patients with unknown micro-organisms (not statistically significant). Antibiotic switching is defined in two ways: changing combined antibiotic therapy to single antibiotic therapy (mono-therapy) or vice versa, and changing an extended spectrum antibiotic therapy for a more pathogen-directed (small-spectrum) antibiotic as shown in Table 3b, antibiotic success defined as continuous mono-therapy or switching from combination therapy to mono-therapy is found more often in the group of patients with known micro-organism (OR 3.17 (CI 1.10-9.17), p = 0.03). Similar was found from switching from extended to pathogen-directed small spectrum antibiotics (OR 3.22 (CI 1.17-8.85), p = 0.02). For patients with

negative pathogen identification, the opposite was found: switching from small-spectrum antibiotics to antibiotics with an extended anti-microbiological spectrum (OR 0.27 (0.08-0.92), p = 0.03).

Table 3a: Relation between outcome and positive identification of causative micro-organism of CAP

Parameter	Micro-organism	
	Unknown (n = 73)	*Known* (n = 128)
In-hospital mortality, n (%)	4 (5)	6 (5)
Admission to ICU, n (%)	7 (10)	21 (10)
Length of hospital stay, mean (SD)	15 (12)	15 (12)

Table 3b: Relation between antibiotic treatment and positive identification of causative micro-organism of CAP

Parameter of antibiotic therapy	Micro-organism		OR (CI), p-value
	Unknown (n = 72)	*Known* (n = 124)	*OR (CI)*
Switch in general, n (%)	29 (40)	60 (48)	NS
Continuous mono-therapy, n (%)	51 (71)	90 (73)	NS
Switch from combination[1] to mono-therapy, n (%)	11 (15)	28 (23)	NS
Continuous combination[1] therapy, n (%)	6 (8)	3 (2)	NS
Switch from mono to combination[1] therapy, n (%)	4 (6)	3 (2)	NS
Antibiotic success[2] - 1, n (%)	62 (86)	118 (95)	3.17 (1.10-9.17), p = 0.03
Continuous small-spectrum[3], n (%)	10 (14)	20 (16)	NS
Switch from extended[4] to small-spectrum[3], n (%)	5 (7)	24 (20)	3.22 (1.17-8.85), p = 0.02
Continuous extended[4] spectrum, n (%)	49 (68)	76 (61)	NS
Switch from small[3] to extended[4] spectrum, n (%)	8 (11)	4 (3)	0.27 (0.08-0.92), p = 0.03
Antibiotic success[5] - 2, n (%)	15 (21)	44 (36)	2.09 (1.06-4.12), p = 0.03

1 Duo- or triple antibiotic therapy

2 Antibiotic success is defined as continuous antibiotic mono-therapy or switching from combination to mono-therapy

3 Small-spectrum antibiotics: beta-lactam antibiotics (penicillin and amoxicillin), first generation cefalosporins, macrolides and doxycyline

4 Extended-spectrum antibiotics: amoxicillin-clavulate, second and third generation cefalosporins, quinolones, co-timoxazol and any combination therapy

5 Antibiotic success is defined as continuous antibiotic therapy with small-spectrum antibiotics or switching from an extended-spectrum to a small-spectrum antibiotic

Discussion

In 64% of the patients, positive pathogen identification of CAP was achieved. Predictors of failure were pre-hospital antibiotic treatment, a medical history of hypertension and a relatively low C-reactive protein at admission (< 300mg/dl). Antibiotic treatment before admission is a well-known risk for failure to identify the causative pathogen of CAP using conventional cultures of blood and sputum [3]. Our results confirm this finding, even if a more extended diagnostic protocol was used compared to previous studies. It is obvious that cultures can become negative if the causative micro-organism (like *Streptococcus pneumoniae*) is sensitive for the antibiotic initiated before admission (in most cases a beta-lactam antibiotic). This does not count for positive viral testing. The finding of a relatively low C-reactive protein in patients in which pathogen identification failed, maybe complementary with the finding that these patients are more often treated with antibiotics before admission, although both predict failure of pathogen-identification independently in multivariate analysis. Another explanation could be that, despite extensive testing for viral and atypical micro-organisms, this group contains more patients with atypical or viral pneumonia (which are often characterized by a low level of C-reactive protein).

A medical history of hypertension as an independent predictor for diagnostic failure in CAP has, by our knowledge, not been reported before. A possible explanation could be that these patients are treated less restrictively with antibiotics by their family doctors. This, in combination with an earlier admission to hospital, could make the load of micro-organism lower, making positive testing by non-invasive testing more difficult. But the frequency of antibiotic therapy before admission in patients with a medical history of hypertension compared to patients without is comparable (respectively 21% and 26%). Another possible explanation could be that patients with a medical history of hypertension are mistakenly diagnosed as suffering from CAP, while they are suffering of heart failure. This is unlikely because these patients have radiological findings comparable with pneumonia and C-reactive protein and WBC-counts above the upper limits of normal in combination with fever. At last, another possibility could be the effect of the use of anti-hypertensive medication, like angiotensin converting enzyme (ACE)- or angiotensin-II (ATII)-inhibitors. Previous studies report ACE-inhibitors as a risk factor for CAP [16-17]. But this only explains the presence of patients with a medical history of hypertension in our group, but is still no explanation for the risk of failure of pathogen-identification.

A limitation of this study is whether a positive test truly identifies the causative pathogen. Negative bacterial tests with a positive viral test may be due to previous treatment by a general practitioner. The antibiotic therapy was, however, in all patients with positive testing directed to the identified pathogen (or stopped in case of positive viral test); failure of antibiotic treatment in this group was low (Table 3b). It seems safe to treat the patient with antibiotics aimed at the positively tested pathogen. Another limitation of this study is, although testing was extensive and done in the majority of the patients, we did not succeed in performing all tests in all patients (especially serologic testing). At last, the number of patients was too small to calculate a more powerful prediction model.
International guidelines state that sputum samples should be taken for culture in all patients with severe CAP, in patients who fail to improve on therapy, and in patients with non-severe pneumonia, who did not receive antibiotic treatment before admission [8-11]. Our study shows that an extensive, non-invasive search for a causative micro-organism reveals pathogen identification in 64% of

the patients with CAP. In addition, we were able to identify patients at risk for failure of pathogen-identification (prior antibiotic treatment, medical history of hypertension and relatively low C-reactive protein). In order to perform pathogen-guided antibiotic therapy, it is important to identify patients at risk for failure of pathogen identification [7]. Pathogen identification is especially important in severe pneumonia and more invasive diagnostic techniques, like bronchoalveolar lavage, may benefit in patients with risk factors for negative pathogen identification. This should be a subject for further research in a larger population.

In conclusion, a causative pathogen was found in about two third of patients hospitalized for CAP. Previous antibiotic treatment, a history of hypertension and a relatively low CRP at admission are indicators of diagnostic failure of pathogen identification. These items should be validated in a larger population.

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Part I: Clinical characteristics

Chapter 3

Prior outpatient antibiotic use as predictor for microbial aetiology of community-acquired pneumonia: hospital based study

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Abstract

Background

Different studies examined age and co-morbidities as predictors for microbial aetiology of pneumonia. We aimed to assess whether receipt of prior outpatient antimicrobial treatment is predictive for microbial aetiology of community-acquired pneumonia.

Methods

This was a hospital based prospective observational study including all patients admitted with community-acquired pneumonia between 1 October 2004 and 1 August 2006. Microbial investigation included sputum, blood culture, sputum PCR, antigen testing, and serology. Exposure to antimicrobial drugs prior to hospital admission was ascertained through community pharmacy dispensing records. Multivariate logistic regression analysis was conducted to assess whether prior outpatient antimicrobial treatment is predictive for microbial aetiology. Patient demographics, co-morbidities and pneumonia severity were considered other potential predictors.

Results

Overall, 201 patients were included in the study. The microbial aetiology was determined in 64% of the patients. The five most prevalent pathogens were *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila. Mycoplasma pneumoniae* and Influenza virus A+ B. Forty-seven of the patients (23%) had received initial outpatient antimicrobial treatment. In multivariate analyses, initial outpatient treatment was associated with a three-fold increased chance of finding atypical pathogens and a three-fold decreased probability of pneumococcal infection. The corresponding odds ratios were 3.11 (95% CI 1.16-8.33) and 0.39 (95% CI 0.17-0.91) respectively.

Conclusion

Prior outpatient antimicrobial therapy is very relevant information in the diagnostic workup aiming to identify the causative pathogen and planning corresponding treatment in patients hospitalised for pneumonia.

Introduction

Community-acquired pneumonia remains a major reason for hospital admission and a common cause of death in developed countries [1, 2]. The initial management of patients hospitalised with pneumonia consists mostly of empirical antimicrobial treatment [3, 4]. Appropriate antimicrobial treatment is essential as inadequate antimicrobial treatment, generally defined as microbial ineffective therapy against the causative pathogen, can influence patient outcome [5]. Because the causative pathogen is not always identified, especially not during the first days after hospitalisation, many studies have focussed on other parameters suggestive for the causative pathogen. Most frequently studied for this purpose are patient characteristics (age and co-morbidities) and severity of pneumonia [6, 7]. Besides this, non-responsiveness to prior outpatient antimicrobial treatment could also act as a predictor for aetiology of pneumonia. The latter, however, has not been extensively studied before. The aim of the present study was to assess whether prior outpatient antimicrobial treatment is predictive for microbial aetiology in patients admitted to hospital for community-acquired pneumonia.

Methods

The study was conducted in the St. Antonius Hospital, a 600-bed teaching hospital (Nieuwegein, The Netherlands).

Patient population

This was a prospective observational study of patients with confirmed pneumonia admitted between October 1, 2004 and August 1, 2006. Pneumonia was defined as a new or progressive infiltrate on a chest X-ray plus at least two of the following criteria: cough, sputum production, temperature > 38°C or < 35°C, ausculatory findings consistent with pneumonia, leucocytosis or -penia (> 10 G/L, < 4 G/L, or > 10% rods in leucocyte differentiation), C-reactive protein > 3 times the upper reference value for normal. Patients, who were immune compromised (systemic steroid use at admission (prednison equivalent > 20 mg/daily for more than 3 days, haematological malignancies and other immunosuppressive therapy) were excluded. The study was approved by the local Medical Ethics Committee and informed consent was obtained from each patient.

Microbial aetiology workup

At least two blood cultures were performed and sputum was taken for Gram-stain and culture and analysed by Taqman real-time PCR for *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydophila* species [8]. Pharyngeal samples were taken for viral culture. Urine was sampled for antigen testing on *Streptococcus pneumoniae* and *Legionella pneumophila* (Binax NOW®) [9, 10]. In addition serum samples of the day of admission and day 10 or 30 were analysed in pairs for detection of a fourfold rise of antibodies to respiratory viruses, *Coxiella burnetii*, *Mycoplasma pneumoniae*, and *Legionella pneumophila* by complement fixation assay [11]. For each patient, the total workup was completed and the microbiology department was blinded for data on outpatient antibacterial drug use. When both viruses and bacteria were identified in a patient, bacteria prevailed for definite aetiology.

Exposure to antimicrobial therapy

Microbial drug use was acquired through community pharmacy dispensing records capturing all drug exposures the year before hospital admission. A patient was considered exposed to an antimicrobial drug when a prescription was filled within 14 days prior to hospitalisation. The name, dosage and amount of antimicrobial drug dispensed were also ascertained. Based on the reigning Dutch guidelines on the initial treatment of patients with suspected pneumonia, the prescribed antimicrobial drug was classified as appropriate or inappropriate [12, 13].

Co-morbidity assessment

Besides outpatient antimicrobial drug use, co-morbidities and other relevant patient characteristics were identified to address factors related with aetiology of community-acquired pneumonia. Co-morbidities were defined based on the presence of conditions for which the patient was under active medical supervision or was receiving treatment at the time of hospital admission. Co-morbidities evaluated were pulmonary diseases (chronic obstructive pulmonary disease, or treated asthma), congestive heart failure, diabetes (both type I and type II), history of stroke, and end-stage renal disease (serum creatinine > 150 μmol/L). Furthermore, patients were classified according to the Pneumonia Severity Index (PSI) developed by Fine et al [14]. In this index patients are classified in five categories representing predicted mortality risk (with the fifth category for highest mortality risk). The outpatient use of oral corticosteroids and gastric acid suppressing drugs was also ascertained.

Statistical analysis

The SPSS statistical package (version 12.0.1 for Windows; SPSS, Chicago, IL) was used for the statistical analyses. Continuous data were expressed as mean ± SD or median (interquartile range) where appropriate. To study the association between prior outpatient antimicrobial treatment and aetiology of pneumonia multivariate logistic regression analyses were applied. Analyses were conducted for overall aetiology and relevant pathogens separately. All baseline characteristics were considered potential confounders. Potential confounders were included in the multivariate model when they were retained after backward stepwise elimination. Significance was set at a p-value < 0.05. The model's performance (goodness-of-fit and discriminative ability) was tested by performing the Hosmer and Lemeshow test and calculating the area under the receiver operator characteristic (ROC) curve.

Results

In total 201 patients with pneumonia were included in the study. The mean age of the patients was 63 (± 17) years and 124 were male. Three patients (1%) were admitted from a nursing home. Clinical symptoms as well as co-morbid illnesses are summarized in Table 1. The overall median duration of hospital stay was 10 days (7-14) and 21 patients were admitted to the intensive care ward. During hospital stay, 10 patients died, all due to pneumonia. The overall 28-days mortality rate was 5%.

Forty-seven patients (23%) had received antimicrobial treatment in the 14 days time-window prior to hospital admission. The antimicrobial drugs dispensed to these patients are summarized in Table 2. The majority of the patients (79%) had their prescription filled within 4 days prior to hospital

admission and 85% of the prescribed antimicrobial drugs complied with the reigning Dutch guidelines [12, 13]. A microbial aetiology could be determined in 128 (64%) of the patients.

Table 1: Demographics, co-morbidities and clinical severity of 201 patients with community-acquired pneumonia

Characteristic	N (%)
Age (years)	
< 60	74 (37)
60 – 69	39 (19)
70 – 79	50 (25)
> 80	38 (19)
Gender	
Male	124 (62)
Female	77 (38)
Co-morbidities	
Pulmonary diseases	71 (35)
Heart failure	18 (9)
Diabetes	35 (17)
History of stroke	17 (9)
End-stage renal disease	10 (5)
Nursing home resident	3 (1)
Co-medication	
Oral corticosteroids	58 (29)
Gastric acid suppressing drugs	61 (30)
*Fine class at admission**	
I	30 (15)
II	34 (17)
III	53 (26)
IV	56 (28)
V	28 (14)

* Fine et al. [14]

Table 2: Outpatient antibiotics utilization profile prior to hospitalisation for community-acquired pneumonia

Type of antimicrobial drug	No. of users (%)*	Appropriate**
Amoxycillin / clavulanic acid	18 (38)	Yes
Amoxycillin	12 (26)	Yes
Doxycycline	7 (15)	Yes
Clarithromycin	5 (11)	Yes
Co-trimoxazole	4 (8)	No
Ciprofloxacin	2 (4)	No
Norfloxacin	1 (2)	No
Azithromycin	1 (2)	Yes

* Total percentage exceeds 100% because some patients (n=3) had two prescriptions

** Based on reigning Dutch guidelines NVALT and SWAB [12, 13]

Table 3 shows the yield of different techniques for the aetiological diagnosis of community-acquired pneumonia. In the population which was hospitalised after prior outpatient antimicrobial treatment, fewer causative pathogens were found compared to patients without prior antimicrobial treatment (57% vs. 66%) (crude OR: 0.71, 95% CI 0.36-1.38). In patients with prior treatment, an aetiology in the group comprising atypical bacterial pathogens was more probable (10 of 47 cases (21%)) (crude OR: 3.51, 95% CI 1.39-8.90), and especially pneumonia due to *Mycoplasma pneumoniae* (5 of 47 cases (11%)) (crude OR: 4.46, 95% CI 1.15-17.37). Aetiology of *Streptococcus pneumoniae* was less prevalent in patients with prior antimicrobial treatment (8 of 47 cases (17%) (crude OR: 0.40, 95% CI 0.18-0.92). In multivariate analyses, these associations remained significant. The associations (both univariate and multivariate) are listed in Table 4. The goodness-of-fit of both multivariate models was excellent with a p-value of 0.966 (Hosmer and Lemeshow test) for the model predicting pneumococcal pneumonia, and a p-value of 0.863 for the model predicting pneumonia of atypical aetiology. The corresponding areas under the ROC curve were 0.64 and 0.79 respectively. In patients aged < 60 years without co-morbidities, aetiology of atypical bacterial pathogens was more prevalent (OR: 4.64, 95% CI 1.72-12.56). Pulmonary co-morbidity was associated with the finding of *Streptococcus pneumoniae* and *Haemophilus influenzae* as causative pathogens (OR 1.87, 95% CI 1.00-3.47 and OR 3.72, 95% CI 1.20-11.57 respectively).

Table 3: Yield of different techniques for the aetiology of community-acquired pneumonia

	Sputum culture	Sputum PCR	Antigen testing	Blood culture	Serology	Viral culture
Number of samples	148	78	183	182	130	88
Number positive	78	14	36	19	38	14
Percentage positive	53	18	20	10	29	16
Streptococcus pneumoniae	33	-	30	17	-	-
Haemophilus influenzae	19	-	-	0	-	-
Legionella pneumophila	1	5	6	-	7	-
Mycoplasma pneumoniae	-	7	-	-	8	-
Staphylococcus aureus	6	-	-	1	-	-
Other Gram-negative bacteria	10	-	-	1	-	-
Other atypical bacteria	-	2	-	-	1	-
Other Gram-positive bacteria	2	-	-	0	-	-
Respiratory viruses	-	-	-	-	22	14

Table 4: Odds ratios (OR) for aetiology and prior outpatient antimicrobial treatment in patients admitted to hospital for community-acquired pneumonia

Aetiology	Prior outpatient antimicrobial treatment		OR (95% CI)
	Yes	*No*	
Total no. of samples	47 (100)	154 (100)	
Univariate			
Pneumococcal	8 (17)	52 (34)	0.40 (0.18 – 0.92)
Atypical	10 (21)	11 (7)	3.51 (1.39 – 8.90)
Viral	4 (9)	12 (8)	1.10 (0.34 – 3.59)
Gram negative strains	3 (6)	20 (13)	0.46 (0.13 – 1.61)
Other	2 (4)	6 (4)	1.10 (0.21 – 5.62)
Unidentified	20 (43)	53 (34)	1.41 (0.73 – 2.75)
Multivariate			
Pneumococcal	-	-	0.39 (0.17 – 0.91) *
Atypical	-	-	3.11 (1.16 – 8.33) **

OR: Odds Ratio; CI: Confidence Interval

* Adjusted for pulmonary diseases and use of oral corticosteroids

** Adjusted for age and pulmonary diseases

Discussion

Our study shows that in patients admitted for community-acquired pneumonia, whether the patient received initial outpatient treatment is associated with a three-fold decreased chance of having an infection with *Streptococcus pneumoniae* and a three-fold increased probability of having pneumonia of atypical aetiology. These findings indicate that information about prior outpatient antimicrobial therapy is very relevant in the diagnostic workup aiming to identify the causative pathogen and planning corresponding treatment in patients with pneumonia.

The initial management of patients hospitalised with pneumonia has been under constant study in different settings during the past decades. Choice of antimicrobial treatment, time to first antimicrobial drug administration, and route of administration all have appeared to be relevant factors in relation to the outcome of pneumonia [15-17]. In light of choice of antimicrobial treatment, knowledge of predominant microbial patterns in CAP represents an essential basis for initial decisions about empirical antimicrobial treatment. In literature, the most frequently found pathogens in community-acquired pneumonia are *Streptococcus pneumoniae*, *Haemophilus influenzae*, Influenza virus A and B, *Legionella pneumophila* and *Chlamydophila* species [3]. The aetiology distribution found in the present study is in accordance with this literature. Because *Streptococcus pneumoniae* is the most frequent pathogen, beta-lactam antibiotics are preferred as initial empirical antimicrobial treatment in treatment guidelines on CAP [3, 13, 18]. Beta-lactam antibiotics, however, do not cover *Legionella pneumophila, Chlamydophila* species, and *Mycoplasma pneumoniae*, the so-called atypical pathogens. Therefore, patients with pneumonia of atypical aetiology treated as outpatient with beta-lactam antibiotics will probably not respond to treatment which could cause deterioration of the situation and lead to subsequent hospital admission. Our finding of an increased prevalence of atypical pathogens in patients with prior outpatient antimicrobial treatment supports such an explanation, but also confirms what has already been suggested in the current British Thoracic Society guideline for the management of community-acquired pneumonia in adults [3, 19]. In this guideline is stated that after failure of initial empirical antibiotic treatment, the microbiological examination should be reassessed with a view to excluding less common pathogens such as atypical pathogens. To our knowledge, the present study is the first study to specifically document failure of initial outpatient antibiotic treatment as predictor for microbial aetiology of community-acquired pneumonia. Another explanation for the observed reduced likelihood of pneumococcal pneumonia in patients who received prior outpatient antimicrobial treatment could be a growth suppression of *Streptococcus pneumoniae* in blood and sputum cultures through the presence of antibiotics. This could mask *Streptococcus pneumoniae* as the causative pathogen. We think, however, that such an explanation is less plausible, especially because we also used antigen testing for identification of the causative pathogen [20]. In addition, such a mechanism can not explain the finding of an increased probability of pneumonia caused by atypical pathogens.

The present study was conducted in a single teaching hospital in the Netherlands, what could pose questions about extrapolation to other hospitals and clinical settings. We think, however, that the external validity of the present study is sufficiently high. First, because the percentage of identified aetiology (64%) in this study is similar to those found in other studies using a similar extent and nature of microbiologic techniques [21-23]. Second, because our patient characteristics very much

comply with a previous nationwide study on prior outpatient antibacterial therapy as prognostic factor for mortality in patients hospitalised for pneumonia [24]. In that large database study, the percentage of patients hospitalised after initial outpatient antimicrobial treatment was almost identical to the percentage observed in the present study (27% vs. 23%). In addition, also age distribution, co-morbidities and outpatient antibiotics utilization profile were very similar as were the median duration of hospital stay and in-hospital mortality. Unfortunately, due to limited numbers, we were unable to study an association between prior outpatient antimicrobial treatment and mortality in the present study.

Besides prior antimicrobial treatment, our study also showed an association between aetiology and age, and pulmonary co-morbidity. Patients aged <60 years without co-morbidities were more likely to have an aetiology comprising viral or atypical bacterial pathogens, and pulmonary co-morbidity was independently associated with *Streptococcus pneumoniae* and *Haemophilus influenzae* as causative pathogens. These findings confirm previous studies on the impact of age, and co-morbidity on microbial aetiology of community-acquired pneumonia [6]. A limitation of the present study, however, is that we were not able to adjust for confounding by smoking habits and alcohol intake of the patients. Previous studies on determinants for pneumonia aetiology also showed that these factors are significant predictors for pneumococcal infection [6, 7]. On the other hand, we do not expect prior antimicrobial therapy and smoking and alcohol intake to coincide in such a way that this would result in finding a null effect when this information was available.

In conclusion, in patients admitted for pneumonia, whether or not a patient has received prior outpatient antimicrobial therapy is very relevant information in the diagnostic workup aiming to identify the causative pathogen and planning initial treatment at the time of hospital admission. This finding supports further strengthening of continuity of care at the interface between the extramural and hospitalised settings.

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Part I: Clinical characteristics

Chapter 4

Clinical and laboratory profiles in pneumococcal and atypical pneumonia

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Submitted

Abstract

Background

The most frequently identified causative micro-organisms of community-acquired pneumonia (CAP) are *Streptococcus pneumoniae*, atypical bacteria and *Haemophilus influenzae*. As antibiotic therapy for these groups of micro-organisms differ, much effort is spent on early identification of these micro-organisms. We studied the additional role of early and easily accessible, clinical and laboratory parameters in the discrimination between these micro-organisms in patients with CAP.

Methods

In 201 adult patients presenting with CAP, routine clinical and laboratory parameters were collected. Identification of pathogens was performed by culture of sputum, blood and pharynx (viral), urine antigen tests, polymerase chain reactions of sputum and serologic testing. We studied differences in clinical and laboratory parameters between the most common causative micro-organisms of CAP.

Results

Patients with pneumococcal pneumonia (n=60; 47%) had higher levels of C-reactive protein (CRP) (median 305 mg/l, IQ 288) than patients with non-pneumococcal pneumonia (median 178 mg/l, IQ 204; p=0.003). Patients with atypical pneumonia (n=21, 16%) had – in contrast to patients with non-atypical pneumonia – higher body temperature (mean 38.8°C, SD 1.1 versus 38.2°C, SD 1.1; p=0.009), lower concentrations of potassium (median 3.5 mmol/l, IQ 0.7 versus 3.9 mmol/l, IQ 0.7; p=0.039) and sodium (mean 133 mmol/l, SD 5 versus 136 mmol/l, SD 4; p=0.046).

Conclusion

We identified different clinical and laboratory profiles for patients with pneumococcal and atypical pneumonia, which may contribute to early pathogen identification.

Introduction

Community-acquired pneumonia (CAP) is most frequently caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* [1-9]. Early identification of the causative pathogen of CAP is helpful for the appropriate choice of antibiotic treatment. Due to the low frequency of penicillin-resistance, *Streptococcus pneumoniae* can still be treated with penicillin in the Netherlands [10]. Antibiotic treatment of *Haemophilus influenzae* consists of amoxicillin (> 90% of *Haemophilus influenzae* is amoxicillin sensitive in the Netherlands). CAP caused by *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Coxiella burnetii* or *Chlamydophila pneumoniae/psittaci* – sometimes called 'atypical pneumonia' – requires for its treatment macrolides (erythromycin) or fluoroquinolones in the case of *Legionella pneumophila*; doxyxcyline is given for *Coxiella burnetii* [9].

Streptococcus pneumoniae can be detected by cultures (sputum, blood or pleural fluid) and by the pneumococcal urine antigen test [5, 8-17]. Early diagnosis of atypical pneumonia is still difficult. A urine antigen test is available for screening of *Legionella pneumophila* serotype 1, but this test is not sensitive for other *Legionella* serotypes, causing 8-12% of Legionella pneumonia [18-20]. Other diagnostic tools to identify the causative micro-organism of CAP are polymerase chain reactions (PCRs) of sputum and serology [5, 8, 11]. Though never conclusive, it could be interesting to use patient characteristics in trying to predict the causative micro-organism of CAP, especially because these characteristics, like clinical and laboratory parameters, are directly available at the moment of presentation in the emergency department.

The aim of this study is to identify the role of early and easily obtainable, clinical and laboratory parameters in the prediction of the most frequently causative micro-organisms in patients with CAP.

Methods

Patients

All patients (>18 years) with CAP presenting in the period October 2004 - August 2006 in a 600-bed teaching hospital in the Netherlands were included in this study. Patients with a history of recent hospitalization (<30 days) or a congenital or acquired immunodeficiency (including patients treated with prednisone ≥20 mg/day for more than 3 days) were excluded. A pneumonia was defined as a new infiltrate on the chest X-ray and two out of six clinical signs of pneumonia (cough, production of sputum, signs of consolidation on respiratory auscultation, temperature >38° or <35°C, leukocytosis (white blood cell count (WBC)>$10x10^9$/l) or leukopenia (WBC<$4x10^9$/l, or more than 10% rods in the differentiation and C-reactive protein (CRP) 3x above the upper limit of normal (5 mg/l). The chest X-ray was interpreted by the resident in the emergency department. The patient was definitely included, if the existence of a new infiltrate on chest X-ray was confirmed by an independent radiologist. The following data were collected at presentation: age, sex, medical history of COPD, mean arterial blood pressure (MAP), heart rate, respiratory rate, temperature, pulse oxygen saturation, haemoglobin, white blood cell count, platelet count, CRP, sodium, potassium, urea, creatinine, albumin, bilirubin, glucose and arterial blood gas. Fine-scores were calculated for each patient. This

study was approved by the local Medical Ethics Committee and informed consent was obtained from each patient.

Pathogen identification

Pathogen identification was performed according to a diagnostic protocol, which has been described before [21]. In short, it consisted of cultures of sputum and blood, urine antigen testing for *Streptococcus pneumoniae* and *Legionella pneumophila*, PCRs of sputum for detection of *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydophila pneumoniae/psittaci* and serologic testing for respiratory viruses, *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Coxiella burnetii*, and viral culture of the pharynx in patients admitted to the hospital during the influenza period. Sputum was regarded as non-representative if Gram-stain showed more than 50 epithelial cells per microscopic view (10 x magnification) or if the number of epithelial cells is between 25-50 cells per view in absence of leukocytes. Urine antigen testing was done by using the Binax *Streptococcus pneumoniae* and Binax *Legionella pneumophila* urine antigen test. Polymerase chain reactions were performed by in house developed Taqman real-time PCR. Blood samples for serologic testing were obtained on the day of admission and day 10 of the hospital stay for detection of antibodies against respiratory viruses, *Mycoplasma pneumoniae* and *Coxiella burnetii* (by complement fixation reaction (CBR). A four fold rise in antibody titres was considered as positive. For the detection of antibodies against *Legionella pneumophila* (IgG/IgM virion/serion ELISA Clindia) blood samples were taken on day of admission and at a control visit at least 30 days after admission. Finally, during the influenza-season viral cultures of the pharynx were done. *Streptococcus pneumoniae* was identified as the causative micro-organism of CAP in case of positive culture of sputum or blood, or positive urine antigen testing, or any combination, *Haemophilus influenzae* in the case of positive cultures of sputum or blood, *Legionella pneumophila* in the case of positive cultures of sputum, positive urine antigen testing, positive PCR or seroconversion, or any combination. *Mycoplasma pneumoniae* was identified as the causative micro-organism of CAP in the case of positive PCR or seroconversion, or any combination, *Chlamydophila pneumoniae*/*psittaci* in the case of positive PCR and *Coxiella burnetii* in the case of seroconversion. Viral pneumonia was only diagnosed in the case of positive viral testing (culture and/or seroconversion) in combination with complete negative bacterial testing (cultures of sputum and blood, PCR, urine antigen testing and serologic testing), otherwise patients were diagnosed as suffering with CAP with an unidentified pathogen. In the case of mixed positive bacterial-viral testing, the patient was diagnosed as suffering with bacterial CAP (with viral co-infection). In the case of mixed bacterial cultures, the bacterium with the most abundant growth was considered as the causative micro-organism of CAP. In the case of mixed bacterial culture and testing (urine antigen testing and/or PCR), the pathogen cultured in the sputum and/or blood was considered as the causative micro-organism within a mixed infection.

Statistical analysis

Statistical analysis was done by using the SPSS 12.0 package. Univariate analysis was first done by one-way ANOVA or Pearson Chi-square tests, followed by Student t-tests or Mann-Whitney tests (dependent on the distribution of data) or Chi-square-tests/Fisher exact tests. Multivariate analysis was done by binary logistic regression analysis. A difference $p<0.05$ was considered as statistically significant.

Results

We considered 255 consecutive patients eligible for this study. In 201 patients, the existence of a new infiltrate on chest X-ray was confirmed. In 128 patients (64%), the causative micro-organism of CAP was identified. Most frequently identified micro-organisms were: *Streptococcus pneumoniae* (n=60, 47%), *Haemophilus influenzae* (n=14, 11%), *Mycoplasma pneumoniae* (n=9, 7%) and *Legionella pneumophila* (n=9, 7%). Other identified micro-organisms were viruses (mainly influenza; n=16, 13%), Gram-stained negative bacteria (mainly *Klebsiella pneumoniae*; n=9, 7%), non-pneumococcal Gram-stained positive bacteria (mainly *Staphylococcus aureus*; n=8, 6%) and other atypical bacteria (*Chlamydophila pneumoniae/psittaci* and *Coxiella burnetii*; n=3, 2%). Mixed infections were found in 24 patients (19%), mainly *Streptococcus pneumoniae* with a virus. Further analysis was done with the groups of patients with CAP caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, atypical bacteria and other causative micro-organisms of CAP.

Demographic, clinical and laboratory characteristics of the four groups of patients with the most frequently identified micro-organisms are shown in Table 1. Differences between groups were found for age, sex, medical history of COPD, WBC, CRP, sodium, potassium, urea, pH, $PaCO_2$ and Fine-score. The first three groups of patients showed statistically significant differences in characteristics; details are described in the next paragraphs. The fourth group of patients with other identified micro-organisms had no specific features. Table 2 shows the differences between CAP caused by *Streptococcus pneumoniae* as compared to patients with non-pneumococcal pneumonia. Patients with pneumococcal pneumonia had significantly higher values for the inflammatory parameters (CRP and WBC), lower platelet count, lower mean arterial blood pressure and higher bilirubin. In the pneumococcal group, CRP value remained statistically higher and low levels of MAP reached the level of statistical significance (p=0.05) in multivariate analysis. For CRP, the area under the curve (AUC) in ROC-curve analysis was 0.66 (CI 0.56-0.76; p=0.002). A CRP>300 mg/l has a sensitivity of 52% and a specificity of 78% for pneumococcal pneumonia.

Table 1: Demographic characteristics, pulmonary medical history, clinical and laboratory variables and Fine-score of 201 patients with (CAP caused by *Streptococcus pneumoniae*, atypical bacteria, *Haemophilus influenzae* and other identified micro-organisms

Parameter	*S. pneumoniae* n=60	Atypical bacteria[1] n=21	*H. influenzae* n=14	Other n=33	*p*
Demographics					
Age (years), median (IQ)	67 (29)	45 (29)	75 (14)	70 (28)	0.003
Males, n (%)	33 (55)	10 (48)	12 (86)	25 (76)	0.028
COPD (%)	22 (37)	2 (10)	9 (64)	11 (33)	0.009
Clinical variables					
Heart rate (b/min), median (IQ)	102 (29)	96 (16)	102 (24)	96 (26)	NS
MAP (mmHg), mean (SD)	90 (15)	95 (12)	100 (15)	96 (17)	NS
Temperature (°C), mean (SD)	38.2 (1.1)	38.8 (1.1)	38.3 (1.2)	38.0 (1.1)	NS
Respiration rate (r/min), median (IQ)	24 (10)	23 (16)	20 (14)	25 (18)	NS
Laboratory variables					
Haemoglobin (mmol/l), mean (SD)	8.2 (0.9)	8.3 (0.9)	8.0 (1.7)	8.1 (1.0)	NS
WBC (10^9/l), median (IQ)	16 (9)	11 (7)	13 (7)	14 (10)	0.04
Platelet count (10^3/l), median (IQ)	252 (121)	265 (115)	225 (132)	290 (188)	NS
C-reactive protein (mg/l), median (IQ)	305 (288)	181 (192)	106 (165)	187 (206)	0.005
Na^+ (mmol/l), mean (SD)	135 (4)	133 (5)	137 (3)	137 (4)	0.009
K^+ (mmol/l), median (IQ)	3.9 (0.7)	3.5 (0.7)	4.3 (0.6)	4.0 (0.5)	0.006
Urea (mmol/l), median (IQ)	8.6 (6.7)	4.4 (4.0)	7.7 (7.1)	8.8 (9.0)	0.043
Creatinine (mmol/l), median (IQ)	102 (62)	73 (30)	115 (56)	84 (46)	NS
Albumin (g/l), mean (SD)	35 (4)	37 (5)	37 (5)	34 (4)	NS
Bilirubin (mmol/l), median (IQ)	16 (8)	11 (8)	12 (14)	14 (11)	NS
Glucose (mmol/l), median (IQ)	6.6 (2.2)	6.2 (3.1)	8.4 (4.2)	7.0 (3.4)	NS
pH, median (IQ)	7.46 (0.05)	7.51 (0.07)	7.44 (0.06)	7.47 (0.07)	0.007
PaCO_2 (kPA),median (IQ)	4.4 (1.2)	4.1 (1.1)	4.7 (1.2)	4.7 (1.1)	0.04
PaO_2 (kPA), median (IQ)	8.7 (2.0)	8.2 (1.2)	10.1 (4.6)	8.9 (3.9)	NS
HCO^{3-} (mmol/l), mean (SD)	23 (3)	23 (3)	24 (3)	25 (3)	NS
O_2- saturated Hb (%), median (IQ)	96 (4)	96 (4)	97 (4)	96 (5)	NS
Fine-score, median (IQ)	81 (52)	70 (48)	116 (59)	95 (58)	0.003

1 Atypical bacteria: *Mycoplasma pneumoniae* (n=9), *Legionella pneumophila* (n=9), *Chlamydophila psittaci/ pneumoniae* (n=2) *and Coxiella burnetii* (n=1)

2 see text for details

Table 2: Characteristics of patients with CAP caused by *Streptococcus pneumoniae* and patients with other causative micro-organism of CAP (non-pneumococcal CAP)

Parameter	*S. pneumoniae* n=60	Other n=68	*p* (univariate)	*p* (log.regression)
CRP (mg/l), median (IQ)	305 (288)	178 (204)	0.002	0.003
WBC (10^9/l), median (IQ)	16 (9)	12 (8)	0.008	NS
MAP (mmHg), mean (SD)	90 (15)	97 (17)	0.016	NS
Platelet count (10^9/l), median (IQ)	252 (121)	268 (148)	0.028	NS
Bilirubin (mmol/l), median (IQ)	16 (8)	13 (8)	0.046	NS

Table 3: Characteristics of patients with CAP caused by atypical bacteria and patients with other causative micro-organism of CAP (non-atypical pneumonia)

Parameter	Atypical bacteria (n=21)	Other (n=107)	*p* (univariate)	*p* (log.regression)
Temperature (°C), mean (SD)	38.8 (1.1)	38.2 (1.1)	0.017	0.009
K^+ (mmol/l), median (IQ)	3.5 (0.7)	3.9 (0.7)	0.001	0.039
Na^+ (mmol/l), mean (SD)	133 (5)	136 (4)	0.030	0.046
Urea (mmol/l), median (IQ)	4.4 (4.0)	8.6 (7.1)	0.000	NS
pH, median (IQ)	7.51 (0.07)	7.45 (0.06)	0.001	NS
Age (years), median (IQ)	45 (29)	69 (23)	0.005	NS
PaCO_2 (kPA), median (IQ)	4.1 (1.1)	4.6 (1.0)	0.008	NS
COPD, n (%)	2 (10)	42 (39)	0.009	NS
Creatinine (mmol/l), median (IQ)	73 (30)	96 (61)	0.012	NS
WBC (10^9/l), median (IQ)	11 (7)	15 (9)	0.013	NS
Bilirubin (mmol/l), median IQ)	11 (8)	15 (9)	0.022	NS

The characteristics of patients with atypical pneumonia versus those with non-atypical pneumonia are shown in Table 3. Patients with atypical pneumonia are young, lack a pulmonary history and present with a relatively high fever, but low WBC, urea, creatinine and bilirubin. This resulted in a significantly lower Fine-score (p=0.004). Arterial blood gas analysis in these patients often shows a respiratory alkalosis. Furthermore, they present with significantly lower concentrations of sodium and potassium as compared to the non-atypical group. After multivariate analysis, only sodium, potassium and body temperature remained statistically significantly different between the two groups (Table 3). The AUC was significant for temperature (AUC 0.66, CI 0.53-0.79) and potassium (AUC 0.28, CI 0.17-0.39), but not for sodium. A temperature >39°C has a sensitivity of 43% and a specificity of 79%, a K^+<3.5 mmol/l has a sensitivity of 57% and a specificity of 83%. The group of patients with atypical pneumonia consisted of patients with CAP caused by *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydophila* species and *Coxiella burnetii* pneumonia.

The 9 patients with pneumonia caused by *Legionella pneumophila* were characterized by high temperature (mean 39.6°C, SD 0.9), alkalosis (median 7.51, IQ 0.07), hypocapnia (*P*aCO2 median 4.1 kPA, IQ 1.3), hypokalemia (median 3.5 mmol/l, IQ 0.7) and no history of COPD (0%). The 9 patients with pneumonia caused by *Mycoplasma pneumoniae* were relatively young (median 38 years, IQ 16) with relatively low heart rate (median 90 b/min, IQ 17), low CRP (median 108 mg/l, IQ 111), low WBC (median $10x10^3$/l, IQ 5) and low urea (median 3.4 mmol/l, IQ 4.4).

Patients with CAP caused by *Haemophilus influenzae* can be characterized as patients with COPD, higher age, lower CRP and higher potassium compared to patients with non-*Haemophilus* CAP (Table 4). Age and medical history resulted in a significantly higher Fine-score (p=0.009).

Table 4: Characteristics of patients with CAP caused by *Haemophilus influenzae* and patients with other causative micro-organism of CAP (non-Haemophilus CAP)

Parameter	*H. influenzae* n=14	Other n=114	*p* (univariate)	*p* (log.regression)
Age (years), median (IQ)	75 (14)	66 (30)	0.013	NS
CRP (mg/l), median (IQ)	106 (165)	233 (231)	0.016	NS
COPD, n (%)	9 (64%)	35 (31%)	0.018	NS
K^+ (mmol/l), median (IQ)	4.3 (0.6)	3.9 (0.7)	0.045	NS

Discussion

This study describes differences in early and easily obtainable clinical and laboratory parameters between patients with CAP caused by *Streptococcus pneumoniae*, atypical bacteria, *Haemophilus influenzae* and other micro-organisms. Patients with pneumococcal pneumonia are characterized by a relatively high CRP as compared to non-pneumococcal CAP patients. In atypical pneumonia, higher body temperatures and lower concentrations of sodium and potassium are found, if correlated with non-atypical CAP patients.

In CAP caused by *Streptococcus pneumoniae* CRP and WBC are significantly higher, whereas platelets and MAP are significantly lower, at presentation compared to patients with non-pneumococcal CAP. In patients with *Streptococcus pneumoniae* CAP, a CRP>300 mg/l has a specificity for pneumococcal pneumonia of 78%. As a consequence, antibiotic regimen of patients presenting with a high (>300 mg/l) CRP should have at least anti-pneumococcal activity, which is a part of international guidelines [9, 11, 22]. There is almost no penicillin resistance for *Streptococcus pneumoniae* in the Netherlands and most therapeutic protocols contain, therefore, penicillin as first line of antibiotic therapy in non-severe CAP in otherwise healthy adults [10, 11]. Although a high CRP is correlated with pneumococcal pneumonia, it cannot be used to justify mono-therapy with penicillin in every case. High CRP-levels are also found in other micro-organisms such as *Legionella pneumophila*. The other distinguishing parameters of pneumococcal pneumonia - high WBC, low platelets and low MAP - lack sufficient specificity to be used in a diagnostic algorithm.

Patients with atypical pneumonia had significantly higher values of body temperature and lower concentrations of sodium and potassium than those with a non-atypical pneumonia. Other features, such as young age, lack of a history of COPD, low WBC, alkalosis, hypocapnia and low levels of creatinine, urea and bilirubin were also more frequent in patients with atypical pneumonia. Younger age has been described previously in patients with atypical (especially *Mycoplasma pneumoniae*) pneumonia. High fever and hyponatremia are classical features of Legionnaires' disease and have been described before, although specific parameters predicting atypical or Legionella pneumonia have not been identified. [23-28]. Alkalosis, which was of respiratory origin, has not been reported before as a typical finding in atypical pneumonia. A possible explanation of respiratory alkalosis in atypical pneumonia is hyperventilation. The observed frequency of respiration was not increased markedly in the patients with atypical pneumonia. This may be due to failure in observation. Respiratory alkalosis due to hyperventilation is often accompanied by an elevated PaO_2; this was, however, not observed in our patients. The absence of elevated PaO_2 may be due to the more diffuse involvement of both lungs in atypical pneumonia. The Fine-scores in patients with atypical pneumonia are low, so there is a fair possibility that these patients will be treated with penicillin or amoxicillin (especially because all cases were non-epidemic). These antibiotics are inappropriate for atypical bacteria [9, 11, 22]. In the case of high temperature, especially in combination with hypokalemia, hyponatremia and/or respiratory alkalosis, one should consider the possibility of an atypical pneumonia.

In our analysis, the results of the group of patients with atypical pneumonia were clustered because of the similarities in antibiotic treatment, e.g. the lack of response to penicillin and beta-lactam antibiotics. However, presentation and clinical course of the micro-organisms in this group is different. We observed only high body temperature as a typical characteristic of Legionella pneumonia, as reported before. In literature, patients with Legionella pneumonia are also correlated with younger age, the use of alcohol, cigarette smoking, prior antibiotic treatment or hospitalization, headache, diarrhoea, lack of medical history or presence of pulmonary history, raised creatine kinase levels, elevated liver enzymes and hyponatremia [23-28]. Data about variables in patients at risk for Mycoplasma pneumonia are scarce. Young age, but also elderly patients, prior antibiotic treatment and exposure to a sick household member are reported as risk factors for *Mycoplasma* pneumonia [23, 24, 27, 28]. In our study, young age, low heart rate, low CRP and a low WBC count are associated with Mycoplasma pneumonia. The numbers of patients in these groups are, however, too small for further calculations and firm conclusions.

So far, guidelines about treatment of CAP do not take into account clinical and laboratory variables in identifying patients at risk for atypical pneumonia, and the syndromic approach to identify the causative micro-organism of CAP has been rejected [9, 11, 22]. The findings in our study suggest that routinely and easily obtainable parameters may play a role in the identification of patients at risk for pneumococcal and atypical pneumonia, though they do not justify the return of the syndromic approach. This study has limitations. It is a single centre study, but the microbiological profile of this group of patients does not differ from previous reports [reviewed in 9]. In our opinion, this is a representative group of patients with CAP. Furthermore, the number of patients with identified micro-organisms other than *Streptococcus pneumoniae* is low.

In conclusion, we found specific profiles of early and easily accessible clinical and laboratory variables in patients with pneumococcal and atypical pneumonia. Larger studies are needed to confirm these findings. It may be of interest to include clinical and laboratory parameters in a diagnostic strategy in patients with CAP.

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Intermezzo: ACE polymorphisms

Chapter 5

Angiotensin-converting enzyme insertion/deletion polymorphism and risk and outcome of pneumonia

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Abstract

Background

Recent studies have suggested involvement of the angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism in the susceptibility to and severity of pneumonia in Asian populations. We have explored the hypothesis that the ACE I/D polymorphism affects the risk and outcome of community-acquired pneumonia in a Dutch white Caucasian population.

Methods

This is a hospital-based prospective observational study including patients with community-acquired pneumonia admitted between October 2004 and August 2006. All patients were genotyped and pneumonia severity and clinical outcome were compared between patients with the II, ID, and DD genotype of the ACE gene. Pneumonia severity was assessed on day of admission and consecutively on day 2, 3, 5 and 10 of hospital stay using APS scoring. Outcomes evaluated were: duration of hospital stay, ICU admittance, in-hospital and 28-days mortality. To study the association between ACE genotype and risk of pneumonia, the distribution of the ACE I/D polymorphism was compared with healthy control subjects from the same geographical region.

Results

In total, 200 patients with pneumonia and 200 control subjects were included in the study. The mean age of the patients was 63 years. APS scores were not different between the genotype groups on any of the days and all clinical outcomes (duration of hospital stay, ICU admittance, in-hospital and 28-days mortality) were comparable between the three genotype groups. The ACE I/D genotype distribution was identical for patients and control subjects (p=0.973).

Conclusion

The ACE I/D polymorphism is not associated with risk and outcome of community-acquired pneumonia in the Dutch white Caucasian population.

Introduction

Community-acquired pneumonia (CAP) ranks in the top-10 leading causes of death with estimated mortality rates varying between 5 and 20% [1, 2]. Despite substantial progress in standards of care and the availability of prediction rule models to identify patients at high risk [3], the mortality rate and impact of pneumonia on health remain high [4, 5]. Therefore, it is considered that, besides demographics and co-morbidities, also genetic factors play an important role in the susceptibility to and severity of pneumonia.

Recently, the involvement of the renin-angiotensin system in the pathogenesis and evolution of pneumonia has gained substantial interest. The use of angiotensin-converting enzyme (ACE)-inhibitors has been associated with lower risk of pneumonia, particularly in elderly patients, and patients using ACE-inhibitors are less likely to die from pneumonia [6-8]. ACE-inhibitors may act on the pathogenesis of pneumonia in two different ways: first, they induce the cough reflex through inhibition of the degradation of the protussive peptides bradykinin and substance P [9, 10], and second, they have an immunemodulatory effect through lowering angiotensin II levels [11-16]. Serum ACE levels are also determined genetically through the identified insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene. The I/D polymorphism has been reported to account for 47% of the variance in serum ACE level, whereas the DD genotype is associated with the highest levels of serum ACE [17].

The ACE I/D polymorphism can also been linked to pneumonia as persons with the DD genotype have a lower cough reflex compared with II and ID [18, 19], and the DD genotype carriers have higher serum levels of the pro-inflammatory angiotensin II [20]. Morimoto et al. already showed that the ACE D allele is an independent risk factor for (fatal) pneumonia in an Asian population [21]. We have explored the hypothesis that the ACE I/D polymorphism affects the risk and clinical outcome of community-acquired pneumonia in a Dutch white Caucasian population.

Methods

Study design and subjects

The study was conducted in St. Antonius Hospital, a 600-bed teaching hospital (Nieuwegein, The Netherlands), and approved by the local Medical Ethics Committee. Informed consent was obtained from each subject. The ethnicity of the population in and around the city of Nieuwegein is primarily (>94%) white Caucasian [22]. This was a prospective observational study of patients with confirmed pneumonia admitted between October 1, 2004 and August 1, 2006. Pneumonia was defined as a new or progressive infiltrate on a chest X-ray plus at least two of the following criteria: cough, sputum production, temperature > 38°C or < 35°C, ausculatory findings consistent with pneumonia, leucocytosis or -penia (> 10 G/L, < 4 G/L, or > 10% rods in leucocyte differentiation), C-reactive protein > 3 times the upper limit of normal. Patients, who were immune compromised (systemic steroid use at admission (prednison equivalent > 20 mg/daily for more than 3 days), hematological malignancies and other immunosuppressive therapy) were excluded.

Microbiological confirmation was sought using sputum for Gram-stain and sputum and blood for

culture. Sputum was analysed by PCR for atypical pathogens (*Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydophila psittaci* and *pneumoniae*). Urine was sampled for antigen testing on *Streptococcus pneumoniae* and *Legionella pneumophila*. In addition, serum samples of the day of admission and day 10 were analysed in pairs for detection of a fourfold rise of antibodies to respiratory viruses, *Coxiella burnetii*, *Mycoplasma pneumoniae*, and *Legionella pneumophila* by complement fixation assay. Pharyngeal samples were taken for viral culture. As control group, ACE I/D genotype data were used from a population of healthy employees of the St. Antonius Hospital who volunteered for venapunction. All control subjects were Dutch white Caucasians. Other characteristics of this population have been described elsewhere [23]. The control subjects did not have a history of pneumonia.

Sample size calculation

In the recent study of Morimoto et al., the relative risk (DD vs. II+ID) was 2.9 for pneumonia and 4.4 for fatal pneumonia [21]. To detect a clinical significant effect of ACE I/D polymorphism on pneumonia outcome we hypothesized that carriers with the DD genotype of the ACE gene would have a 3-fold increased mortality risk compared with carriers of the II and ID genotype. Considering a baseline mortality risk of 10% combined with 25% DD genotype carriers, this resulted in a estimated sample size of 196 patients (α=0.05, power=0.80). For the effect of the ACE polymorphism on the susceptibility for pneumonia, considering a relative risk for pneumonia of 2 for carriers of the DD genotype compared with II+ID [21], the required sample size to detect a significant effect of genotype on pneumonia risk was estimated at 153 patients and 153 control subjects (α=0.05, power=0.80). Beforehand, the aim of the present study was set at the inclusion of 200 patients and 200 control subjects.

Outcome measures and illness severity assessment

The following outcome measures were identified for all patients: duration of hospital stay, the need for intensive care admittance, survival to hospital discharge, and 28-days mortality. To quantify illness severity the APS (Acute Physiology Score) score was calculated for each patient on admission and consecutively on day 2, 3, 5 and 10 of hospital stay [24]. In addition, for each patient the highest APS score during hospital stay and the occurrence of ARDS (Acute Respiratory Distress Syndrome) was identified.

Genotyping

Genomic DNA of patients was isolated from EDTA blood using the MagNA Pure LC DNA Isolation kit 1 (MagNA Pure; Roche Diagnostics). ACE I/D polymorphisms were determined by real-time PCR using fluorescent hybridization probes and a LightCycler (Roche Diagnostics) as described earlier with some slight modifications [17, 25, 26]. Briefly, the reaction volume was 20 μl, containing 1 μl of DNA (40-80 ng), 0.2 μM forward primer and 0.8 μM reversed primer reported by Rigat et al. [17], 2 μl of 10x reaction buffer (LightCycler DNA master hybridization probes, Roche Diagnostics), 1.6 μl of 25 mM $MgCl_2$ stock solution and 0.1 μM of each probes. The detection probes were the same as described by Somogyvari et al. [26]. The PCR conditions were as follows: denaturation at 95°C for 60s, followed by 50 cycles denaturation (95°C for 10s), annealing (first 10 cycles: 67°C for 20s, followed by 0.5°C stepwise decrease per cycle to 61°C) and extension (72°C for 30s). Melting curve analysis consisted of heating to 95°C for 5s, 45°C for 60s, followed by an increase of the

temperature to 75°C at 0.2°C/s. To exclude mistyping of I/D heterozygotes as D/D homozygotes, a second PCR reaction was conducted under the same conditions except for using the primer pair as described earlier [26, 26]. Verification of the real-time PCR results with those of electrophoresis and using SSP-PCR revealed no mistyping. ACE I/D polymorphisms were determined after follow-up of the patients, excluding confounding by indication.

Co-morbidity assessment

Besides ACE genotyping, co-morbidities were identified to address factors related with outcome in community-acquired pneumonia. Co-morbidities were defined based on the presence of conditions for which the patient was under active medical supervision or was receiving treatment at the time of hospital admission. Co-morbidities evaluated were lung diseases (chronic obstructive pulmonary disease, or treated asthma), congestive heart failure, diabetes (both type I and type II), and end-stage renal disease (serum creatinine > 150 μmol/L). Furthermore, patients were classified according to the Pneumonia Severity Index (PSI) developed by Fine et al [3]. The use of ACE-inhibitors and angiotensin-II receptor blockers was also assessed.

Statistical analysis

The SPSS statistical package (version 12.0.1 for Windows; SPSS, Chicago, IL) was used for the statistical analyses. Continuous data were expressed as mean ± SD or median (range) where appropriate. Categorical data were analysed by chi-square and continuous data by Student's t tests, rank tests, and one-way analysis of variance where appropriate. Multivariate logistic regression analyses were applied to study the association between ACE genotype and need for ICU admittance, in-hospital mortality, and 28-days mortality. All baseline characteristics were considered potential prognostic factors for clinical outcome. Non-significant variables ($p>0.05$) were removed stepwise from the model. The chi-square tables were used to compare the observed number of each genotype with those expected for a population in Hardy-Weinberg equilibrium and to compare genotype frequencies between the patients with pneumonia and the control subjects. For all tests, a p-value of ≤ 0.05 was considered significant.

Results

In total 201 patients with pneumonia and 200 control subjects were included in the study. For one patient a DNA sample was missing, leaving 200 patients and 200 control subjects eligible for further analysis. There were no major differences in demographics and clinical characteristics of the patients by ACE genotype (Table 1). Based on microbiological data the patients were categorized as pneumococcal pneumonia, atypical pneumonia, pneumonia with Gram-negative strain, viral pneumonia, or aetiology unknown. In total, aetiology was available for 127 patients (64%). Aetiology was not different for the three ACE genotype groups (Table 1).

The overall median duration of hospital stay was 9.5 days and 21 patients were admitted to the intensive care ward (Table 2). During hospital stay, 10 patients died, all due to pneumonia. The overall 28-days mortality rate was 5.0% and not statistically different between the three ACE genotypes (7.1%, 3.8%, and 5.8% for II, ID and DD respectively; p=0.668). The mean highest APS score dur-

ing hospital stay was 23.9 and not statistically different between the genotype groups. There was no trend towards an association between ACE genotype and risk of ARDS.

Figure 1 shows the mean APS scores during the episode of pneumonia by ACE genotype. Using one way analysis of variance, the scores were not statistically different on any of the days (p=0.350). None of the patients in the low PSI risk classes (risk class I-II) died during hospital stay. For the patients with a moderate (risk class IV) or high risk (risk class V), the in-hospital mortality rates were 5.4% and 22.2% respectively. In univariate analysis, the risk class at admission was significantly associated with in-hospital mortality (p<0.01). In the multivariate analyses, no associations between ACE I/D polymorphism and need for ICU admittance, in-hospital mortality, nor 28-days mortality could be detected as ACE genotype did not reach significance in any of the models. When ACE genotype (DD vs. II+ID) was added to the final model afterwards, this yielded odds ratios of 0.81 (95% CI 0.15-4.29), 1.53 (95% CI 0.34-6.86), and 0.47 (95% CI 0.12-1.77) for respectively in-hospital mortality, 28-days mortality, and ICU admittance. Exclusion of patients using ACE-inhibitors or angiotensin II receptor blockers from the analyses did not cause a change in the findings (data not shown).

Table 1: Baseline characteristics of 200 patients with community-acquired pneumonia by ACE I/D polymorphism

	all n=200	II n=42	ID n=106	DD n=52	p-value
Demographics					
Age (SD)	63 (17)	61 (17)	65 (17)	60 (18)	0.224
Male sex (%)	124 (62)	20 (48)	70 (66)	34 (65)	0.097
Co-morbidity					
Renal disease	10 (5)	2 (5)	4 (4)	4 (8)	0.567
CHF	18 (9)	3 (7)	9 (9)	6 (12)	0.734
Diabetes	34 (17)	7 (17)	20 (19)	7 (14)	0.695
Lung diseases	70 (35)	18 (43)	38 (36)	14 (27)	0.264
ACE/ATII use	43 (22)	7 (17)	28 (26)	8 (15)	0.197
Etiology					0.402
Pneumococcal	60 (30)	17 (41)	30 (28)	13 (25)	
Atypical	21 (11)	5 (12)	11 (10)	5 (10)	
Viral	16 (8)	3 (7)	11 (10)	2 (4)	
Gram-negative strain	22 (11)	6 (14)	9 (9)	7 (14)	
Other	8 (4)	0 (0)	4 (4)	4 (8)	
Unknown	73 (37)	11 (26)	41 (39)	21 (40)	
*Risk class**					0.252
Low I	30 (15)	7 (17)	15 (14)	8 (15)	
Low II	34 (17)	8 (19)	16 (15)	10 (19)	
Low III	53 (27)	4 (10)	35 (33)	14 (27)	
Moderate IV	56 (28)	17 (40)	25 (24)	14 (27)	
High V	27 (13)	6 (14)	15 (14)	6 (12)	

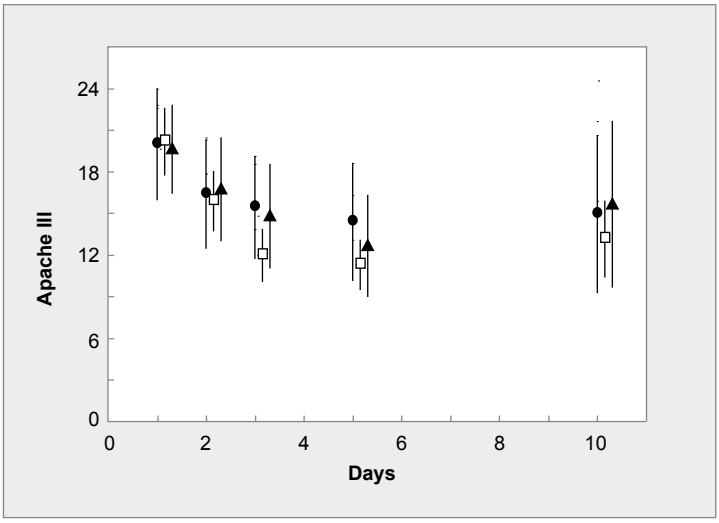
* Pneumonia Severity Index based on Fine et al. [3]

Table 2: Clinical outcomes and illness severity by ACE I/D polymorphism

	II n=42	ID n=106	*DD* n=52	p-value
Clinical outcomes				
Duration of stay [median(range)]	11.5 (4-49)	9 (2-143)	9 (3-59)	0.548
ICU admittance [n(%)]	7 (17)	11 (10)	3 (6)	0.230
Days on ICU [median(range)]	5 (1-13)	8 (3-64)	4 (4-16)	0.282
In-hospital mortality [n(%)]	2 (5)	6 (6)	2 (4)	0.883
28-days mortality [n(%)]	3 (7)	4 (4)	3 (6)	0.668
Illness severity				
APS [mean (SD)]*	26 (13)	23 (12)	23 (13)	0.350
ARDS [n(%)]	1 (2)	3 (3)	0 (0)	0.481

* mean calculated based on highest score for each individual

Figure 1: Mean APS scores (APACHE II) with 95% confidence intervals by ACE genotype (● = II, □ = ID, ▲ = DD) on day of hospital admission (day 1) and during hospital stay (days 2,3,5, and 10).



For both patients with pneumonia and control subjects, the ACE I/D genotype distribution was compatible with the Hardy-Weinberg equilibrium. The genotype and allele frequencies did not differ between patients and control subjects (Table 3).

Table 3: Genotype and allele frequencies of the ACE I/D polymorphism

	pneumonia n=200	controls n=200	p- value
Genotype			
II	42 (21)	43 (22)	0.973
ID	106 (53)	107 (54)	
DD	52 (26)	50 (25)	
Allele			
I	190 (48)	193 (48)	0.832
D	210 (52)	207 (52)	

Discussion

In this hospital based prospective observational study, no differences in clinical development of community-acquired pneumonia were observed between patients with the DD, ID, and II genotype of the ACE gene. Furthermore, there was a similar distribution of genotypes and allele frequencies of the ACE I/D gene in patients with pneumonia and control subjects, suggesting no association between the ACE I/D polymorphism and the risk of acquiring pneumonia.

The recent study of Morimoto et al. [21] found that the ACE D allele was an independent risk factor for pneumonia in elderly patients with a relative risk of 2.9 (95% CI 1.7-4.8). Although our study had sufficient power to detect an odds ratio of 1.60 or more, we were not able to confirm this association. A major difference between the present study and the study from Morimoto et al. is the ethnicity of the population under study. Morimoto studied Japanese patients solely, whereas we studied a Dutch white Caucasian population. Reports have shown marked ethnic differences between polymorphisms of the renin-angiotensin system components, especially of the ACE gene [27]. The prevalence of the DD genotype is small in Asian populations compared to white and African populations [28]. When we compare the ACE I/D genotype frequencies between our control group and the control group of Morimoto we can confirm this difference (frequency DD genotype: 25% *versus* 11%, $p<0.001$). The genotype frequency of our control group is very much in line with other white Caucasian control groups published in literature [29-30]. The ACE I/D polymorphism also occurs in multiple haplotypes. Possibly, the ACE I/D polymorphism is not a functional polymorphism but rather a marker for a true functional polymorphism for which the linkage dysequilibrium with the true functional polymorphism is different between ethnic groups.

Another difference between the present study and the study of Morimoto is the setting of the study. Morimoto studied elderly inpatients in a long-term care hospital because of the known increased risk of pneumonia due to aspiration in this group of patients. Furthermore, they studied patients only for the non-winter months in order include mostly aspiration events. Increased risk of aspiration through decreased activity of the cough reflex via decreased local levels of the protussive peptides bradykinin and substance P is proposed as one possible mechanism responsible for the effect of ACE D

allele on pneumonia risk [18]. We think, however, that this difference cannot explain the finding of a null effect in the present study. Firstly, when we limited our analysis to non-winter (April-November) events the genotype distribution of the patients remained identical to that of the control subjects (II/ID/DD: 21/50/24; p=0.929). Secondly, the genotype distribution of the patients did not differ with age.

At last, the finding of a null effect could also be explained by the inclusion of admitted pneumonia cases solely. Approximately 60% of patients with pneumonia is treated at home. Therefore, one might argue that admission itself is dependent on genotype, either because those of one genotype (i.e. DD) die before referral or because those of one genotype (i.e. II) are not sufficiently unwell to be admitted. Such admission bias, however, seems unlikely given that genotype distribution is in Hardy-Weinberg equilibrium and very similar to our control group.

Besides no association between ACE I/D polymorphism and pneumonia risk, our study also showed no association between the ACE I/D polymorphism and pneumonia outcome. The pneumonia illness severity, as quantified by APS, was not different for the three genotype groups as was the duration of hospital stay, in-hospital mortality, and 28-days mortality. Recently, Harding et al. showed that the ACE D allele is associated with increased risk of organ dysfunction and death with meningococcal meningitis [31]. Another study from Marshall et al. suggested an important role for the ACE I/D polymorphism in the susceptibility and outcome in ARDS [32]. This was partly confirmed by Jerng et al. concluding that the ACE I/D polymorphism is a prognostic factor for the outcome of ARDS [33]. Also Adamzik et al. showed an association between the ACE DD genotype and increased 30-day mortality in ARDS [29]. In the present study, we were not able to extend these findings to community-acquired pneumonia. There was no trend towards an association between ACE I/D polymorphism and the occurrence of ARDS in our patients (Table 2). This is in accordance with the previous findings from Jerng et al. and Adamzik et al. [29, 33]. Due to limited numbers we were unable to examine the association between ACE I/D polymorphism and outcome in ARDS.

Regarding the association between the ACE I/D polymorphism and clinical outcome of community-acquired pneumonia, we realize that the numbers of outcomes in the present study were smaller than expected and that this could explain the finding of a null effect due to lack of power. On the other hand, the absence of any trend towards an effect of ACE DD genotype on pneumonia outcome makes the need for additional studies with larger numbers questionable. Also a subgroup analysis (data not shown) in patients with confirmed pneumococcal pneumonia showed no trend of an effect of ACE genotype on disease severity. Our study still had sufficient power to detect a 10% absolute difference in mortality between the DD and ID+II genotypes.

In conclusion, according to our findings, the ACE gene I/D polymorphism is not associated with risk and outcome of community-acquired pneumonia in the Dutch white Caucasian population.

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Intermezzo: ACE polymorphisms

Chapter 6

ACE I/D corrected serum ACE activity and severity assessment of community-acquired pneumonia

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Abstract

Background

Different studies described decreased serum angiotensin-converting enzyme (ACE) activity in patients with pneumonia. The present study aimed to evaluate the role of ACE in pneumonia by comparing ACE insertion/deletion (I/D) genotype corrected serum ACE activity and to establish whether the severity of the disease correlates with lower ACE activity.

Methods

This was a prospective hospital-based observational study including 134 patients with pneumonia. Serum ACE activity was determined at admission, on day 2, 3, 5 and 10 of hospital stay, and at recovery. Based on ACE genotype and reference values, corresponding Z-scores were calculated. Disease severity, quantified by the Acute Physiology Score (APS), and clinical outcome were compared between tertile groups of the Z-scores.

Results

A significant decrease in serum ACE activity during an episode of pneumonia with return to control range during recovery was observed for all 3 genotypes (II, ID and DD). The calculated Z-scores showed a negative correlation with APS scores ($p=0.050$). No significant association between decreased serum ACE activity and clinical outcome could be observed.

Conclusion

Serum ACE activity is significantly decreased during the acute phase of pneumonia. Despite correction for ACE I/D genotype, decreased ACE activity did not show a prognostic value. Further studies are suggested on the mechanisms behind and diagnostic value of a decreased ACE activity in community-acquired pneumonia.

Introduction

Community-acquired pneumonia remains a major reason for hospital admission and a common cause of death in developed countries [1, 2]. Therefore, pneumonia is subject to many studies on demographic variables, co-morbidities, and biological markers in order to predict outcome and to evaluate a patients' management.

The renin-angiotensin system is a feedback regulated system. In response to a fall in blood pressure renin is secreted into the circulation. Renin cleaves angiotensinogen to generate angiotensin I. Angiotensin I has no appreciable activity, but is acted on by a second proteolytic enzyme, angiotensin-converting enzyme (ACE) (peptidyldipeptidase A, EC 3.4.15.1) to form the highly active angiotensin II. The majority of ACE is expressed on the surface of (pulmonary) endothelial cells [3] and leaked into the circulation from ACE-expressing cells by proteolytic cleavage [4]. Circulating concentrations of ACE have been extensively studied in relation to different human lung disorders [5, 6]. Today, one quite established value of serum ACE measurements is in the diagnosis and follow-up of sarcoidosis [7, 8]. Furthermore, serum ACE has also been studied in patients with adult respiratory distress syndrome (ARDS) and pneumonia where ACE activity showed a strong decrease in the acute phase of the disease with return to control range within a few days [9-13].

Although proposed, so far no relation between these alterations in serum ACE activity and clinical development of both ARDS and pneumonia could be established [14]. One possible explanation for this could be the large inter-patient variations in serum ACE activity observed in these studies. Nowadays, the identified ACE insertion/deletion (I/D) polymorphism, accounting for almost half of the variance in serum ACE activity, provides an explanation for these inter-patient variations but also a need to re-investigating the possible clinical value of serum ACE activity in pneumonia by considering ACE I/D genotype [15].

The objective of the present study was to evaluate the role of ACE in community-acquired pneumonia by comparing the genotype corrected serum ACE activity and to establish whether the severity of the disease correlates with lower ACE activity.

Methods

The study was conducted in St. Antonius Hospital, a 600-bed teaching hospital (Nieuwegein, The Netherlands). The ethnicity of the population in and around the city of Nieuwegein is primarily (>94%) white-Caucasian [16].

Patient population

This was a prospective observational study of patients with confirmed pneumonia admitted between October 1, 2004 and August 1, 2006. Pneumonia was defined as a new or progressive infiltrate on a chest X-ray plus at least two of the following criteria: cough, sputum production, temperature > 38°C or < 35°C, ausculatory findings consistent with pneumonia, leukocytosis or -penia (> 10 G/L, < 4 G/L, or > 10% rods in leukocyte differentiation), C-reactive protein > 3 times the upper limit of

the reference interval for normal values. Patients, who were immunocompromised (systemic steroid use at admission (prednisone equivalent > 20 mg/daily for more than 3 days), haematological malignancies and other immunosuppressive therapy) or who were using ACE-inhibitors, angiotensin II receptor blockers, or aldosterone antagonists were excluded. All patients were required to sign an informed consent and the study was approved by the Ethics Committee of the St. Antonius Hospital. In total, 158 patients with pneumonia were included in the study. For twenty-four of these patients, no blood sample for ACE activity measurement was collected at time of hospital admission and an appropriate DNA sample was missing for one patient. Finally, 134 patients were eligible for further analysis.

Determination of ACE activity

Blood samples were collected aseptically into lithium heparin tubes on admission and on day 2, 3, 5 and 10 of hospital stay. At least 30 days after the resolution of the acute infection, the patients were requested to visit the out-patient clinic to provide another blood sample. Quantification of ACE activity was measured in lithium heparin plasma using the Bühlmann ACE kinetic test, according to previously described methods (Bühlmann Laboratories AG, Switzerland) [17, 18]. The manufacturers' reference interval is 12-68 U/L.

Genotyping

Genomic DNA of patients was isolated from EDTA blood using the MagNA Pure LC DNA Isolation kit 1 (MagNA Pure; Roche Diagnostics). ACE I/D polymorphisms were determined by real-time polymerase chain reaction (PCR) using fluorescent hybridization probes and a LightCycler (Roche Diagnostics) as described earlier with some slight modifications [15, 19, 20]. Briefly, the reaction volume was 20 μl, containing 1 μl of DNA (40-80 ng), 0.2 μM forward primer and 0.8 μM reversed primer reported by Rigat et al. [15], 2 μl of 10x reaction buffer (LightCycler DNA master hybridization probes, Roche Diagnostics), 1.6 μl of 25 mM $MgCl_2$ stock solution and 0.1 μM of each probes. The detection probes were the same as described by Somogyvari et al. [20]. The PCR conditions were as follows: denaturation at 95°C for 60s, followed by 50 cycles denaturation (95°C for 10s), annealing (first 10 cycles: 67°C for 20s, followed by 0.5°C stepwise decrease per cycle to 61°C) and extension (72°C for 30s). Melting curve analysis consisted of heating to 95°C for 5s, 45°C for 60s, followed by an increase of the temperature to 75°C at 0.2°C/s. To exclude mistyping of I/D heterozygotes as D/D homozygotes, a second PCR reaction was conducted under the same conditions except for using the primer pair as described earlier [19, 20]. Verification of the real-time PCR results with those of electrophoresis and using sequence-specific primer PCR revealed no mistyping. ACE I/D polymorphisms were determined after follow-up of the patients.

Genotype corrected ACE activity

After genotyping, all serum ACE activities were translated into Z-scores. The Z-score was calculated as $(ACE_{patient} - \text{mean } ACE_{reference\ group}) / SD_{reference\ group}$ where $\text{mean}_{reference\ group}$ and $SD_{reference\ group}$ are calculated from the ACE values measured in previously described II, ID and DD reference groups originating from the same geographical region as the patients [21]. This reference group consisted of healthy employees of the St. Antonius Hospital who volunteered for venapunction.

Pathogen identification

At least two blood cultures were performed and sputum was taken for Gram-stain and culture and analysed by Taqman real-time PCR for *Mycoplasma pneumoniae, Legionella pneumophila* and *Chlamydophila pneumoniae* and *psittaci* [22]. Pharyngeal samples were taken for viral culture. Urine was sampled for antigen testing on *Streptococcus pneumoniae* and *Legionella pneumophila* (Binax NOW®) [23, 24]. In addition serum samples of the day of admission and day 10 were analysed in pairs for detection of a fourfold rise of antibodies to respiratory viruses*, Coxiella burnetii*, *Mycoplasma pneumoniae*, and *Legionella pneumophila* by complement fixation assay [25]. Based on the findings, patients were classified as pneumonia with bacterial origin, viral pneumonia, or pneumonia with unknown aetiology.

Outcome measures and illness severity assessment

The following outcome measures were identified for all patients: duration of hospital stay, the need for intensive care unit (ICU) admittance, survival to hospital discharge, and 28-days mortality. Illness severity was quantified by Acute Physiology Score (APS) scoring on admission and consecutively on day 2, 3, 5 and 10 of hospital stay [26].

Co-morbidity assessment

Besides demographic variables, co-morbidities were identified to address factors related with outcome of community-acquired pneumonia. Co-morbidities were defined based on the presence of conditions for which the patient was under active medical supervision or was receiving treatment at the time of hospital admission. Co-morbidities evaluated were lung diseases (chronic obstructive pulmonary disease (COPD), or treated asthma), congestive heart failure, diabetes (both type I and type II), and end-stage renal disease (serum creatinine > 150 μmol/L). Furthermore, patients were classified according to the Pneumonia Severity Index (PSI) developed by Fine et al [27]. In this index patients are classified in five categories representing predicted mortality (with the fifth category for highest mortality risk).

Statistical analysis

The SPSS statistical package (version 12.0.1 for Windows; SPSS, Chicago, IL) was used for the statistical analyses. Continuous data were expressed as mean ±SD or median (interquartile range) where appropriate. Categorical data were analysed by chi-square and continuous data by Student's t tests, rank tests, and one-way analysis of variance where appropriate. To study the association between serum ACE and severity of disease a correlation coefficient between Z-score and APS was calculated. Besides this, first, a multivariate linear regression analysis was conducted to identify determinants for serum ACE activity (using ACE activity at recovery), and subsequently, a linear regression analysis was conducted with APS as dependent and serum ACE activity and all parameters that appeared independent determinants for serum ACE activity included in the model. The prognostic usefulness of serum ACE activity was studied in two ways. First, clinical outcomes were compared between tertile-based groups of the distribution of the Z-scores. Secondly, a statistical analysis was focused on the ability of serum ACE activity to predict the outcome of pneumonia. For this purpose, logistic regression models adjusted by the comorbidities, ACE genotype and including serum ACE activity were constructed. The relative risk for having a certain outcome was estimated by odds ratio (OR) and 95% confidence interval (CI). For all tests, a p-value < 0.05 was considered significant.

Results

The mean age of the patients was 61 years (±19) and 79 (59%) of the patients were male (Table 1). On day of hospital admission, the serum ACE activity was significantly different for the II, ID and DD genotype groups with mean serum ACE activities of 24, 28 and 39 U/L respectively (Table 2). In total 96 patients visited the out-patient clinic for a recovery sample. The reason for not visiting was either death (n=8) or lost to follow up (n=30). The ACE genotype distribution did not differ between both time points (II/ID/DD at time of admission was: 32/63/39; at time of recovery: 25/48/23 (p=0.733)) and the genotype distribution was similar to that of the reference group (43/107/50; p=0.356).

When analysed as paired sample (n=96) the serum ACE activity differed significantly between admission and recovery in all three genotype groups (Table 2). The decrease in serum ACE activity was most evident in carriers of the DD genotype. Figure 1 shows the serum ACE activity on day of admission, on day 2, 3, 5, and 10 of hospital stay and at recovery for the three genotype groups. The serum ACE activity of the reference group is also presented in Figure 1. The mean serum ACE activities at hospital admission were 29, 29, and 34 U/L for pneumonia with bacterial origin (n=81), viral pneumonia (n=12), and pneumonia with unknown aetiology (n=41) respectively.

The overall median duration of hospital stay was 9 days and 14 patients were admitted to the intensive care ward. During hospital stay, 8 (6%) patients died, all due to pneumonia. Based on the calculated Z-scores, the patients were divided into three groups of equal size (based on tertiles). This resulted in groups with Z-scores from -3.2 to -1.2 (n=46), from -1.2 to -0.4 (n=43), and from -0.4 to 3.2 (n=45). Duration of hospital stay, in-hospital and 28-days mortality as well as intensive care admittance was not statistically different between the three groups (Table 3). However, there was a trend towards increased disease severity with lower Z-scores. The mean APS score was the highest in the group of patients with lowest Z-scores and APS showed a negative correlation with the calculated Z-scores (p=0.050). In the multivariate linear regression analysis, serum ACE was not significantly associated with APS (p=0.156). Serum ACE activity at recovery was significantly determined by ACE genotype and lung diseases (COPD and asthma). In the multivariate logistic regression analyses, no associations between serum ACE activity and need for ICU admittance, in-hospital mortality nor 28-days mortality could be detected as the serum ACE activity did not reach significance in any of the models. The ACE I/D polymorphism also showed no association with need for ICU admittance and both in-hospital and 28-days mortality (data not shown).

Table 1: Demographic and general characteristics of the study population

Characteristic		n=134
Demographics	Age (SD)	61 (19)
	Male sex (%)	79 (59)
Co-morbidity	Renal disease	8 (6)
	CHF	10 (8)
	Diabetes	17 (13)
	Lung diseases	48 (36)
ACE genotype (II/ID/DD)		32/63/39
Risk class*	Low I	25 (19)
	Low II	24 (18)
	Low III	30 (22)
	Moderate IV	40 (30)
	High V	15 (11)

* Risk class based on Fine et al.

Table 2: Serum ACE activity (U/L) according to the ACE genotype on admission and at recovery

	II	ID	DD	p-value°
ACE acute (n=134)				
Mean (SD)	24 (9)	28 (12)	39 (15)	<0.001
ACE recovery (n=96)*				
Mean (SD)	27 (7)	37 (12)	52 (17)	<0.001
p-value #	0.019	<0.001	<0.001	

* number of patients in acute and recovery group vary in size because not all patients visited the out-patient clinic for a recovery sample (8 patients died and 30 lost to follow-up)

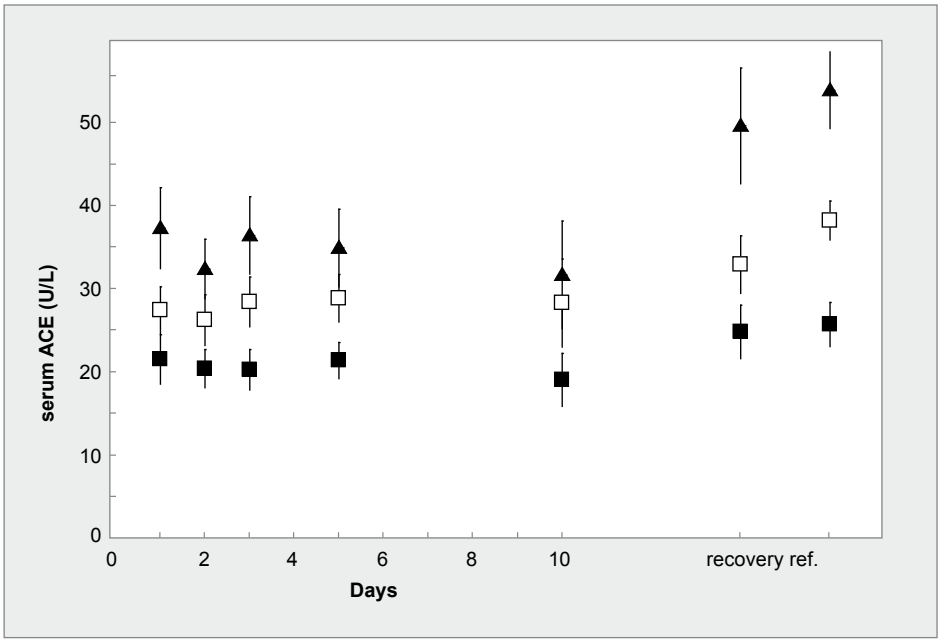
\# paired-sample t-test (n=96)

° one-way analysis of variance

Table 3: Clinical outcomes and illness severity by Z-score tertiles (Z-score calculated based on serum ACE activity at time of hospital admission)

	Z-score			
Severity and outcome	(-3.2 to -1.2) n=46	(-1.2 to -0.4) n=43	(-0.4 to 3.2) n=45	p-value
APS [mean(SD)]	22 (14)	19 (10)	17 (12)	0.111
Duration of stay [median(interquartile range)]	10 (6-15)	9 (7-14)	8 (6-15)	0.908
ICU admittance [n(%)]	5 (11)	3 (7)	6 (13)	0.618
In-hospital mortality [n(%)]	3 (7)	2 (5)	3 (7)	0.906
28-days mortality [n(%)]	3 (7)	2 (5)	3 (7)	0.906

Figure 1: Mean serum ACE activity with 95% confidence intervals on admission (day 1) and on day 2, 3, 5 and 10 of hospital stay, and after recovery for different genotypes (● = II, □ = ID, ▲ = DD). The last column represents the ACE activity of the reference (ref.) group



Discussion

In accordance with the previous findings from Kerttula et al. and Altshuler et al. our study showed a significant decrease in serum ACE activity during an episode of community-acquired pneumonia with return to control range during recovery. The decrease was evenly pronounced in pneumonia with bacterial origin, viral pneumonia, as pneumonia with unknown aetiology. Despite correction for

ACE I/D genotype and a significant correlation with the APS, we were not able to establish a prognostic value for the decreased ACE activity on the outcome of pneumonia.

The pathophysiological mechanisms behind the decrease in serum ACE activity are still unclear. Considering that ACE in the peripheral blood is identical to that produced by the pulmonary endothelial cells, one possible explanation could be an attenuated enzyme release from damaged pulmonary vascular endothelium. The observation from Altshuler et al. that ACE activity decreased more in patients with polysegmentated pneumonia is supportive to such a mechanism [28]. Another possible explanation could be an increased demand for angiotensin II leaving a depleted ACE pool. Hilgenfeldt et al. previously observed higher angiotensinogen levels combined with lower ACE levels in patients with sepsis [29]. The fact that in the present study serum ACE activity also correlated with mean arterial pressure (used in the calculation of the APS score) is supportive to such a mechanism [26]. A third explanation could be the concomitant presence of circulating endogenous inhibitors. This, however, was studied by Altshuler et al but not observed.

In addition to the studies from Altshuler and Kertulla, we included the ACE I/D polymorphism in the association between decreased serum ACE activity and severity of pneumonia but we were still unable to show a solitaire prognostic value for decreased serum ACE activity during the active phase of pneumonia. However, although non significant, our findings show a trend towards a negative correlation between ACE activity and disease severity. Based on these findings we think that further studies with larger numbers are warranted to explore a prognostic value for decreased ACE activity in community-acquired pneumonia. We realize, however, that pneumonia severity is multifactor determined which could preclude clinical significance for the decreased ACE activity itself. The severity and clinical outcome remain strongly determined by demographic patient characteristics and co-morbidities as well as the pneumonia aetiology and antibacterial treatment. To further study the mechanism responsible for the observed decreased serum ACE activity during an episode of pneumonia, concomitant angiotensin II sampling would be helpful.

Although in the present study we were unable to establish a prognostic value for the decreased ACE activity in the acute phase of pneumonia, there might be a diagnostic applicability. To explore this possibility we compared the serum ACE activity at admission with those of the healthy control subjects used for our calculation of the Z-scores. For example, considering a cut point of 52 U/L for patients with the DD genotype for deciding about the diagnosis of pneumonia, ACE activity showed a sensitivity of 52% and a specificity of 90%. These data indicate that an ACE activity above this cut point practically excludes the diagnosis of pneumonia. We realize, however, that such a diagnostic applicability requires further study especially because this was not the primary aim in the present study. Furthermore, also serum ACE activity of initially suspected pneumonia that is not confirmed at follow-up will need to be assessed.

There are some possible limitations to our study. As it was conducted in admitted patients, less severe episodes of pneumonia attended normally in the primary care setting could not be included, although 59% of the episodes were grouped into the low-risk classes (class I-III). Secondly, one could argue about the sample size of the present study. Beforehand, no solid power calculation was conducted as decrease in serum ACE activity was not predictable. Besides this, the mixed character

(e.g. different aetiologies) of the study population could have weakened the power to detect an association between serum ACE activity and outcome of pneumonia. In a post hoc power calculation: our study had sufficient power to detect a three-fold increased in-hospital mortality for patients with a Z-score below -2 compared to patients with higher Z-scores (α0.05; power (1-β):0.80). Another possible limitation is the lack of information about smoking status. Smoking is associated with both an increased risk of pneumonia and increased plasma ACE levels [30]. Therefore, smoking could modify the decrease in serum ACE activity during pneumonia. Unfortunately, it was not possible to evaluate this in the present study. At last, we observed a decrease in circulating concentrations of ACE and this may not necessarily represent a decrease of ACE levels in the lung. Although ACE is mainly derived from endothelial cells and the lung represents the body's largest endothelial surface, in ARDS patients an increased ACE activity has been reported in bronchoalveolar lavage fluid, despite a decrease in circulating concentrations [31].

In conclusion, serum ACE activity is significantly decreased during the acute phase of pneumonia with return to normal during recovery. Despite correction for ACE I/D genotype, the decrease in ACE activity did not show a prognostic value. Further studies are suggested on the mechanisms behind and diagnostic value of a decreased ACE activity in community-acquired pneumonia.

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Part II: Innate immunity

Chapter 7

Interleukin-6 serum levels and polymorphisms in community-acquired pneumonia

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Abstract

Background

Interleukin-6 (IL-6) is a pro-inflammatory cytokine which acts as an acute phase protein. Serum levels of IL-6 are increased in inflammatory diseases and associated with severity of disease. Within the interleukin 6 (IL6) gene, two single nucleotide polymorphisms have been extensively studied: -174 G/C and -572 G/C. We investigated IL-6 serum levels in relation to causative micro-organism and clinical outcome in patients with community acquired pneumonia (CAP). The role of IL6 polymorphisms on susceptibility to and outcome of CAP was also studied.

Methods

A consecutive group of 255 adult patients without congenital or acquired immune-deficiency presenting with CAP, were found eligible for this study. DNA could be genotyped in 200 patients and 311 controls. IL-6 serum levels were measured at admission, on several sequential days of hospital stay, and at a control visit.

Results

IL-6 serum levels were significantly higher in CAP caused by *Streptococcus pneumoniae* (median 449 pg/ml), as compared to other micro-organisms (median 110 pg/ml). IL-6 serum levels, but not C-reactive protein and white blood cell count, were related with ICU-admission, severe sepsis and positive blood cultures. Patients with severe CAP had more frequently the IL6 -174 CC genotype (8/27; 30%), as compared to controls (48/311; 15%).

Conclusion

High IL-6 serums levels (>1020 pg/ml) can be observed in patients with CAP caused by *Streptococcus pneumoniae*. IL-6 is more valuable to predict clinical outcome than CRP or WBC in patients with CAP. The IL6 -174 CC genotype appeared to be an independent risk factor for severe CAP.

Introduction

Community-acquired pneumonia (CAP) is the number one infectious disease requiring hospitalization in Europe [1]. CAP is caused by a wide variety of micro-organisms with *Streptococcus pneumoniae* and *Haemophilus influenzae* as the most frequently cultured micro-organisms [2]. Inflammatory responses, including the cytokine response as measured by interleukin-6 (IL-6), differ between infections with several of these respiratory pathogens. These differences might be used to differentiate between specific micro-organisms causing CAP, and to predict severity of disease.

IL-6 is an acute phase protein within the innate immune system with pro- and anti-inflammatory properties. Pro-inflammatory function consists of delayed apoptosis and enhanced cytotoxicity of neutrophils, T-cell proliferation, development of antigen-specific T-lymphocytes, B-cell stimulation and activation of coagulation pathways [3-6]. Anti-inflammatory effects of IL-6 are the inhibition of the pro-inflammatory cytokines TNFα and IL-1 [7, 8]. In mice, IL-6 is essential for elimination of bacteria from the pulmonary compartment and in its absence (knock out mice models) mortality of pneumococcal airway infection is higher [9-11]. *Streptococcus pneumoniae* and its lipopolysaccharide stimulate IL-6 production in vitro and in vivo [12-16]. IL-6 levels are primarily elevated in the lung, but also in the systemic circulation in human bacterial pneumonia and sepsis [17-20].

Two single nucleotide polymorphisms (SNPs) with in the promotor region of the IL6 gene have been identified: IL6 -174 G/C and IL6 -572 G/C. The functional relation between IL6 -572 SNPs and IL-6 serum levels is unknown, but the presence of the IL6 -174 GC and GG genotypes results in increased IL-6 transcription and production after induction with LPS [21-23]. The effect of these SNPs on susceptibility and outcome in patients with CAP is unknown.

We investigated the usefulness of IL-6 measurements to predict causative micro-organisms and outcome of CAP. We analysed the relation between IL-6 levels and the IL6 -174 G/C and -572 G/C polymorphisms and report on the role of these polymorphisms in susceptibility and outcome.

Methods

Patients and controls

All patients (> 18 years) with CAP hospitalized in the period October 2004 - August 2006 in a general 600-bed teaching hospital (St. Antonius Hospital, Nieuwegein, the Netherlands) were included in this study. Patients with a history of recent hospitalization (< 30 days) or a congenital or acquired immunodeficiency (including the use of corticosteroids more than 20mg prednisone each day) were excluded. Pneumonia was defined as a new infiltrate on the chest X-ray, confirmed by an experienced radiologist, and two out of six clinical signs of pneumonia (cough, production of sputum, temperature >38°C or <35°C, white blood count (WBC) >10x10^9/l or <4x10^9/l or >10% rods in differential count and C-reactive protein (CRP) >15 mg/l). Clinical and laboratory data were collected at presentation, on day 2, 3, 5 and 10 of hospitalization and at a control visit at least 30 days after admission. Fine-score was calculated at the moment of presentation [24]. Clinical and laboratory variables of the APACHEII and SAPSII score systems were scored to calculate severity of disease

during hospitalization [25, 26]. The control group comprised 311 healthy, unrelated, Dutch Caucasians. The study was approved by the local Medical Ethics Committee and written informed consent was obtained from each patient.

Pathogen identification

Sputum and blood of all patients was cultured. Polymerase chain reactions (Taqman real-time PCR) were performed in sputum in order to detect DNA of micro-organisms causing atypical pneumonia (*Mycoplasma pneumoniae*, *Legionella pneumophila* and *Chlamydophila pneumoniae/psittaci*) [27]. Urine samples were used for antigen testing on *Streptococcus pneumonia* and *Legionella pneumophila* serogroup 1 (Binax *Streptococcus pneumoniae* and *Legionella pneumophila* urine antigen test) [28, 29]. *Mycoplasma pneumoniae*, *Coxiella burnetii*, *Legionella pneumophila* or respiratory viruses was detected by serological assays (Complement fixation and IgG/IgM virion/serion ELISA, Clindia, Leusden, the Netherlands) [30]. Pharyngeal samples were taken for viral culture.

Cytokine-assay

We measured serum levels of IL-6 on day 1 (day of admission) and subsequently in serum samples drawn at 8 AM on day 2, 3, 5, 10 and 30. Blood samples were drawn into pyrogen-free vials and within 1 hour the plasma was separated by centrifugation and frozen at -80 °C. Serum samples were available of respectively 168 (84%), 147 (74%), 146 (73%), 155 (78%), 92 (48%) and 121 (61%) patients. The concentration of IL-6 was determined by multiplex immunoassay using a 10-plex human cytokine kit from BioRad Laboratories (Hercules, CA, USA). The assay was performed according to the manufacturer's instruction and run and analyzed on a Bio-Plex 100 Suspension Array System. Lower limit of range of IL-6 measurements was 5 pg/ml and upper limit $1x10^4$ pg/ml.

Genotyping of the IL6 -174 G/C (rs1800795) and -572 G/C SNPs (rs1800796)

From all patients blood was taken at admission and collected in 10 cc containers. A 200 µl whole blood sample was used to extract DNA, using the MagNA Pure LC DNA isolation kit I (Roche Diagnostics, Basel, Switzerland). The extracted genomic DNA was genotyped on an Applied Biosystems TaqMan® 7500 Fast Real-time PCR system with customized primers (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). All runs consisted of a 10 minute incubation period at 95°C followed by 40 cycles of 15 sec at 95°C and 1 minute at 60°C. Afterwards the samples were cooled down. In one patient (an 80 year old female with a Gram-negative Fine-class V pneumonia) DNA extraction failed. Frequencies of SNPs, in patients and controls, were in Hardy-Weinberg equilibrium ($p>0.05$).

Statistical analysis

Statistical analysis was performed using SPSS 12.0 (SPSS Inc, Chicago, IL, USA). Descriptive values were denoted as mean and standard deviation or median and range. The significance of differences of IL-6 serum levels between groups were calculated by Mann-Whitney or ANOVA-tests, or paired sample t-tests in the case of repeated testing within cases. In case of statistically significant findings in univariate analysis, logistic regression and ROC-curve analysis were performed. Statistical analysis of SNP frequencies were performed using Chi-square contingency table analysis.

Results

Initially, 255 consecutive patients were found eligible for this study. In 201 patients, the existence of a new infiltrate on chest X-ray was confirmed by a radiologist unaware of the clinical course of the patient. These patients were included. Base-line characteristics of all patients are shown in Table 1. In 128 patients (64%) the causative micro-organism of CAP was identified. The most frequently identified micro-organism was *Streptococcus pneumoniae* (n=60; 30%), followed by *Haemophilus influenzae* (n=14; 7%), *Legionella pneumophila* (n=9; 5%) and *Mycoplasma pneumoniae* (n=9; 5%). Other micro-organisms were *Staphylococcus aureus* (n=6; 3%), Gram-negative stained bacteria (n=9; 5%, mainly *Klebsiella pneumoniae*), rare atypical pathogens (n=3; 1%), streptococcus species (n=2; 1%) and respiratory viruses (n=16; 8%). In 73 (36%) patients no causative pathogen was identified.

Table 1: Baseline characteristics at admission of 201 patients with CAP

Characteristic		
Male sex, n (%)		124 (62)
Age, mean (min-max)		63 (18-98)
Antibiotic therapy before admission, n (%)		48 (24)
Corticosteroid therapy before admission, n (%)		35 (17)
Micro-organism, n (%)	*Streptococcus pneumoniae*	60 (30)
	Haemophilus influenzae	14 (7)
	Legionella pneumophila	9 (5)
	Mycoplasma pneumoniae	9 (5)
	Other	36 (18)
	Unknown	73 (36)
Fine-class, n (%)	I	30 (15)
	II	34 (17)
	III	53 (26)
	IV	56 (28)
	V	28 (14)

IL-6 serum levels and causative micro-organism of CAP

The IL-6 serum levels in patients grouped according to most frequently identified causative micro-organisms. IL-6 serum levels differed significantly between these groups (ANOVA $p<0.001$). The highest IL-6 serum levels were found in patients with *Streptococcus pneumoniae* pneumonia (median 449 pg/ml, range 6-1x10^4 pg/ml) and differed significantly from the IL-6 serum levels in patients with pneumonia caused by another pathogen (median 110 pg/ml, range <5-10^4 pg/ml, $p<0.001$). Area under the curve in ROC-curve analysis for IL-6 at admission as a predictor for CAP caused by *Streptococcus pneumoniae* is 0.72 (CI 0.63-0.81; $p<0.001$). A cut-off level of >1020 pg/ml has a specificity of 92% for pneumococcal pneumonia (sensitivity of 40%). Other micro-organisms identified in patients with an IL-6 level >1020 pg/ml were Gram-negative bacteria (n=2/30, 3%) and *Legionella pneumophila* (n=1/30, 2%). In multivariate analysis, containing known risk factors for pneumococcal pneumonia (age, COPD), patients with an IL-6 serum level >1020 pg/ml at admission are at risk for CAP caused by *Streptococcus pneumoniae*: OR 8.77 (CI 3.53-21.74; $p<0.001$).

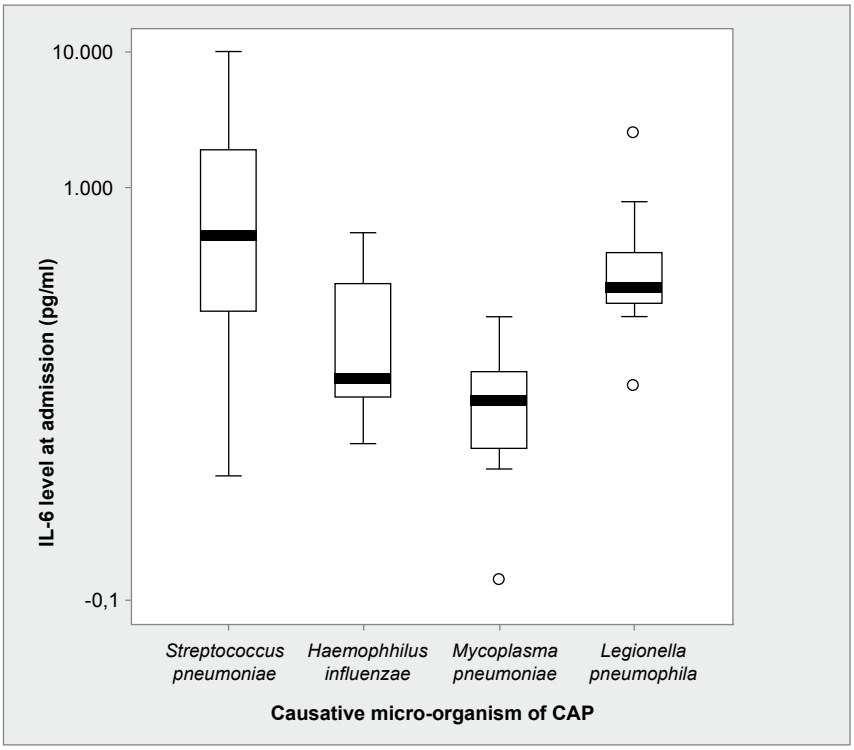
Serum levels of IL-6 were significantly lower in patients with Mycoplasm*a* pneumonia (median 28 pg/ml, range <5-112 pg/ml), compared to patients with CAP caused by other micro-organisms. The number of patients was too small to allow further analysis. In patients with *Haemophilus influenzae*, a tendency was found for low IL-6 levels at admission (median 38 pg/ml, range 12-464 pg/ml; p=0.053).

The variation in IL-6 serum levels at the time of admission was not correlated with age and time between onset of complaints (cough, sputum, fever or dyspnoea) and hospital admission, and did not differ for sex, medical history or Fine-class.

IL-6 serum levels during hospitalization

The highest IL-6 serum levels were found at the moment of presentation and decreased during the following two days of hospitalization. We observed a slight increase in IL-6 serum levels on day 5; this might be the result of the selection of very ill patients who remained hospitalized. Differences between the mean IL-6 levels on consecutive days were significant (ANOVA p<0.005). After admission, the IL-6 level rose in patients with CAP caused by *Mycoplasma pneumoniae*, in contrast to patients with pneumococcal, *Haemophilus influenzae* and Legionella pneumonia. Median IL-6 serum day 2/IL-6 serum day 1 ratios were respectively 0.16 (range 0.01-9.00) in pneumococcal pneumonia, 0.41 (0.03-1.79) in *Haemophilus influenzae* pneumonia, 0.44 (0.16-0.94) in Legionella pneumonia and 1.09 (0.23-93.29) in pneumonia caused by *Mycoplasma pneumoniae*.

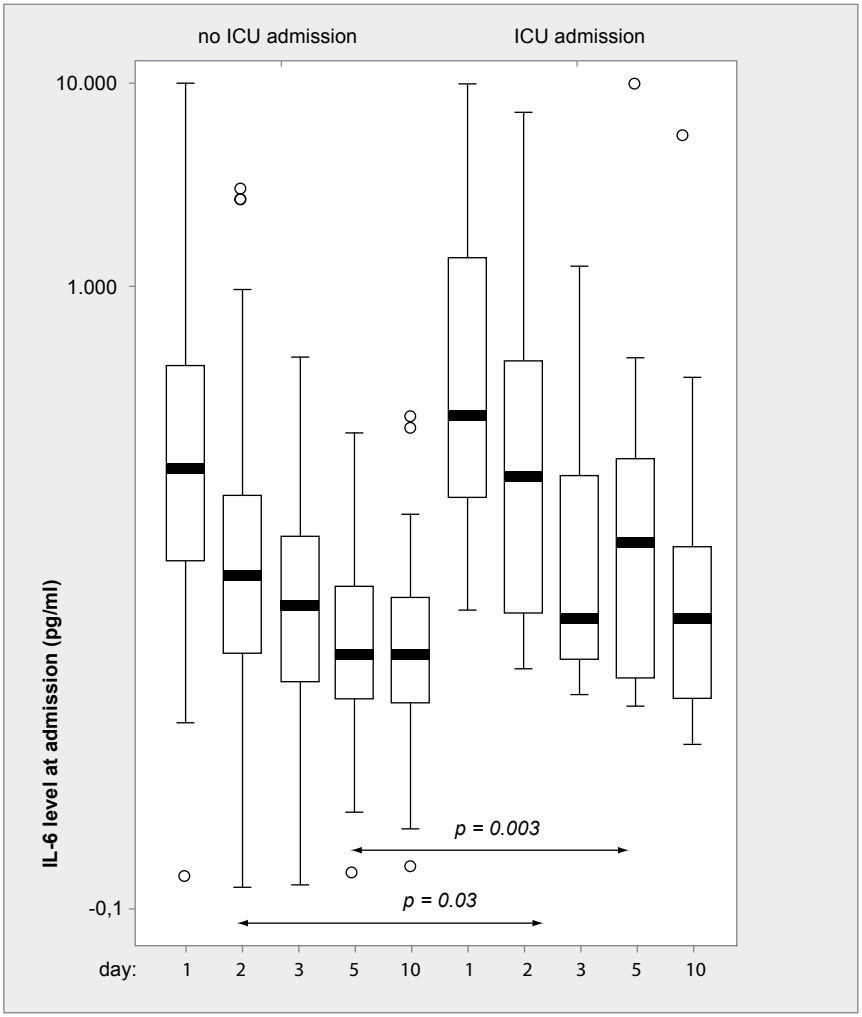
Figure 1: IL-6 serum levels at admission for patients with CAP caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila* or *Mycoplasma pneumoniae*



IL-6 serum levels and outcome

IL-6 serum levels were correlated with in hospital death on day 10 of hospitalization. Seven patients died after day 10. IL-6 serum levels of these patients were significantly higher (median 76 pg/ml, range 9-5598 pg/ml, compared to 15 pg/ml, range 0-360 pg/ml in survivors; AUC 0.81 (CI 0.61-1.00); p<0.01). A cut-off IL-6 level on day 10 of hospitalization >17.5 pg/ml predicted CAP associated mortality with a sensitivity of 86% and specificity of 59%. On day 5 levels were already increased in these patients, but these differences did not reach the level of statistical significance in multivariate analysis. IL-6 serum levels at initial presentation were higher in patients admitted to the ICU during their hospital stay compared to patients who did not require ICU admission (median 239 pg/ml, range 25-10^4 pg/ml and 129 pg/ml, range <5-1x10^4 pg/ml respectively; AUC 0.64; CI 0.51-0.77; p=0.05). As illustrated in Figure 2, IL-6 serum levels were higher in the group of patients admitted to the ICU at every data point. Statistically significant differences were found on day 2 (p=0.03) and day 5 of hospitalization (p=0.003).

Figure 2: IL-6 serum levels at admission and during hospitalization in patients without and with admission to the ICU



Within the group of patients with CAP caused by *Streptococcus pneumoniae*, IL-6 levels at admission were not associated with ICU admission or decease during hospitalization. However, on day 5 of hospital stay, IL-6 serum levels were higher in patients, who (were going to be) admitted to the ICU. Length of hospitalization was only weakly associated with IL-6 levels at admission (correlation coefficient 0.15; $p<0.05$).

IL-6 serum levels were strongly associated with invasive disease. In patients with positive blood cultures, higher IL-6 levels were found at admission (median 986 pg/ml as compared to 136 pg/ml, logistic regression $p < 0.05$, AUC 0.75 (CI 0.63-0.87), $p<0.005$). This was also observed on day 2 of hospitalization. Patients with severe sepsis (within the first 10 days of hospitalization) had significantly higher IL-6 serum levels at admission (logistic regression $p<0.05$, AUC of 0.68 (CI 0.59-0.77; $p<0.001$) as compared to patients with non-severe sepsis. IL-6 serum levels on day 2, 3 and 5 of hospitalization were also significantly higher in patients with severe sepsis. IL-6 serum levels at admission were significantly correlated with clinical scores on day 1 and consecutive days of hospital stay (Fine-score, modified APACHEII and SAPSII). IL-6 serum levels sampled on day 2 and 3 of hospitalization significantly correlated with clinical scores calculated on the same day (modified APACHEII and SAPSII). In patients with pneumococcal pneumonia, IL-6 serum levels were associated with severe sepsis at admission and invasive disease (positive blood cultures) on day 2 and 5 of hospitalization ($p<0.05$).

IL-6 serum levels compared to CRP and WBC

IL-6 serum levels did not correlate with conventional parameters of inflammation such as temperature and WBC. IL-6 serum levels were significantly correlated to CRP (Correlation coefficient 0.16; $p<0.05$). In contrast to serum IL-6, CRP (analysed in the same blood samples as IL-6) was not associated with any clinical outcome, including ICU admission, bacteraemia or severe sepsis. Therefore, IL-6 might be a better marker of severity of disease than CRP.

IL6 promoter SNPs, IL-6 levels and susceptibility to CAP

Table 2 shows the genotype frequencies of the SNPs IL6 -174 and -572 in patients and controls. In one patient, a female with a Fine-class V Gram-negative pneumonia, DNA analysis failed. Genotype frequencies did not differ between patients and controls. Subgroup analysis revealed no differences in IL6 genotype frequencies between the most frequently identified micro-organisms and controls. Within patients, no differences were found in frequency of IL6 SNPs for sex, age, medical history, pre-hospital antibiotic or corticosteroid therapy. Of the 27 patients with the Fine-class V CAP, the IL6 -174 CC genotype was present in 8 (30%) patients, compared with the IL6 -174 GG or GC genotype in 19 (70%) patients. This difference is significant with an OR of 3.86 (CI 1.47-10.15; $p<0.01$). The -174 CC genotype was thereby identified as a risk factor for the presentation with Fine-class V in CAP patients. We did not find differences in IL-6 levels between the different IL6 -174 and -572 genotypes or haplotypes, nor did we find associations between IL6 genotypes and IL-6 serum levels in patients with CAP caused by *Streptococcus pneumoniae*.

Table 2: Genotypes of IL6 promoter SNPs -174 G/C and -572 G/C in controls and patients with CAP, and patients subdivided by causative pathogen and Fine class

SNP	IL-6 -174 G/C, n (%)			IL-6 -572 G/C, n (%)		
Allele	GG	GC	CC	GG	GC	CC
CAP (n = 200)	83 (42)	92 (46)	25 (13)	184 (92)	15 (8)	1 (1)
Streptococcus pneumoniae (n = 60)	28 (47)	21 (35)	11 (18)	55 (92)	5 (8)	0
Haemophilus influenzae (n = 14)	6 (43)	5 (36)	3 (21)	14 (100)	0	0
Legionella pneumophila (n = 9)	4 (44)	5 (56)	0	9 (100)	0	0
Mycoplasma pneumoniae (n = 9)	4 (44)	4 (44)	1 (11)	8 (100)	1 (11)	0
Fine-class I (n = 30)	9 (30)	15 (50)	6 (20)	28 (93)	2 (7)	0
Fine class II (n = 34)	17 (50)	14 (41)	3 (9)	31 (91)	2 (6)	1 (3)
Fine class III (n = 53)	28 (53)	22 (42)	3 (6)	46 (87)	7 (13)	0
Fine- class IV (n = 56)	22 (39)	29 (52)	5 (9)	52 (93)	4 (7)	0
Fine class V (n = 27)	7 (26)	12 (44)	8 (30)*	27 (100)	0	0
Controls (n = 311)	113 (36)	150 (48)	48 (15)	283 (91)	28 (9)	0

* The frequency of the IL6 -174 CC genotype is significantly higher in patients with CAP with Fine-class V (OR 3.86; CI 1.47-10.15; p<0.0038).

IL6 promoter SNPs and outcome

The effect of the IL6 promoter genotypes on clinical course is shown in Table 3. We found no differences for mortality, ICU admission or bacteraemia. The IL6 -174 C allele was more frequently found in patients with severe sepsis, but this difference did not reach the level of statistical significance (OR 1.80, CI 0.93-3.48; p=0.08). Length of hospital-stay did not differ between the different genotypes. Subgroup analysis of the four main groups of micro-organisms revealed no differences between genotypes on outcome. There were no differences in clinical scores between the promoter SNPs during hospital stay.

Table 3: Frequencies of IL6 promoter SNPs -174 G/C and -572 G/C in non-survivors, patients admitted to the ICU, patients with severe sepsis and patients with positive blood cultures

SNP:	IL6 -174 G/C, n (%)			IL6 -572 G/C, n (%)		
Allele	GG	GC	CC	GG	GC	CC
	(n = 83)	(n = 92)	(n = 25)	(n = 184)	(n = 15)	(n = 1)
Non-survivors (n = 10)	4 (40)	4 (40)	2 (20)	9 (90)	1 (10)	0
Admission to ICU (n =21)	10 (48)	10 (48)	1 (5)	20 (95)	1 (5)	0
Severe sepsis (n = 54)	17 (32)	29 (54)	8 (15)	51 (94)	3 (6)	0
Positive blood culture (n = 18)	8 (44)	7 (39)	3 (17)	16 (89)	2 (11)	0

Discussion

The highest IL-6 levels at admission were found in patients with CAP caused by *Streptococcus pneumoniae* and in patients with a complicated clinical course (ICU admission, positive blood cultures and severe sepsis). IL-6 serum levels had a good correlation with clinical course, in contrast to CRP and WBC. The IL6 -174 CC genotype was found to be a risk factor for severe CAP (Fine-class V) and the C allele was associated with severe sepsis.

Initial IL-6 levels were high in patients with *Streptococcus pneumoniae* and low in patients with *Mycoplasma pneumoniae* CAP. In mice and healthy volunteers, a fast increase in IL-6 levels has been observed after infection with *Streptococcus pneumoniae* previously, especially at the site of infection (lung or nasopharynx). High IL-6 levels were also associated with severity of infection. IL-6 levels remained elevated for a period of 7 days in these studies [9, 11, 14, 31]. In adults, IL-6 serum levels in Mycoplasma pneumonia have not been reported before. In children, IL-6 serum levels are low in CAP caused by *Mycoplasma pneumoniae* compared to *Streptococcus pneumoniae* and are dependent of duration and severity of disease [32, 33]. Despite the small number of patients with *Mycoplasma pneumoniae*, we also observed low levels of IL-6 in them. Infection with *Mycoplasma pneumoniae* in mice did also result in a limited local IL-6 response [34]. We observed different dynamics of IL-6 response for various micro-organisms. Patients with CAP, caused by *Streptococcus pneumoniae*, present with high serum levels of IL-6 followed by a fast decrease in IL-6 levels during hospitalization. A similar pattern is found in patients with Legionnaires' disease, who had less prominent IL-6 levels at presentation as compared to pneumococcal pneumonia. However, patients with CAP caused by *Mycoplasma pneumoniae* showed an initial increase in IL-6 serum levels during the first days of hospitalization. *Mycoplasma pneumoniae* is characterized by low IL-6 levels throughout the whole period of hospitalization.

We found a relation between IL-6 levels and severity of CAP (mortality, ICU admission, severe sepsis and positive blood cultures). IL-6 levels as a predictor of clinical outcome of CAP in adults have been reported before [35-40]. In our study, IL-6 at presentation was more accurate than CRP and WBC to predict clinical outcome. High IL-6 serum levels at presentation could therefore be used to identify patients at risk for ICU admission and for severe sepsis.

The number of reports on the role of IL6 promoter SNPs in susceptibility to CAP and its clinical course is limited. The IL6 -174 G/C promoter SNP was of no risk for CAP in general in a previous study [41]. The IL6 -174 CC genotype was identified as a risk factor for severe CAP. This genotype is also associated with other severe infectious diseases, like extra-pulmonary dissemination of pneumococcal disease, especially meningitis, and sepsis [42]. The IL6 -174 CC genotype has been associated with an increased risk for shock in sepsis and possible survival of sepsis after trauma (higher in patients with the IL6 -174 GG genotype) [43-45]. We also found a weak association between the IL6 -174 C allele and severe sepsis. However, others found no relation between the IL6 -174 CC genotype and risk for sepsis after trauma and mortality or multi-organ failure in sepsis.[44, 46, 47] The IL6 -174 CC and IL6 -572 GC genotypes in children were found more often in those admitted to the ICU compared to controls [43]. We observed no relation between IL6 -572 genotypes

and risk for or outcome of CAP in adults. In our opinion, the IL-6 promoter SNPs is, therefore, no risk factor for the susceptibility to CAP. However, patients with the IL6 -174 CC had a higher chance for severe CAP.

In conclusion, IL-6 may be useful to differentiate between pneumococcal and non-pneumococcal pneumonia. ICU admission and severe sepsis are preceded by high IL-6 serum levels. IL-6 serum levels reflect clinical course more accurately compared to CRP and WBC. IL6 promoter SNPs are not a risk for CAP in general, but patients with the IL6 -174 CC genotype have a higher risk for severe CAP.

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Part II: Innate immunity

Chapter 8

IL-10 haplotypes are associated with community-acquired pneumonia in younger patients

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Abstract

Background

Interleukin-10 (IL-10) is an important cytokine in the host response to community-acquired pneumonia (CAP). IL-10 is necessary to counterbalance proinflammatory cytokines, but overexpression might increase pneumonia severity. The aim of this study was to further investigate the role of IL-10 in CAP.

Methods

Systemic IL-10 response was analysed in 201 consecutive patients with CAP. Serum IL-10 concentrations were measured in blood collected at the day of hospital admission and after 2, 3, 5 and 10 days and at a control visit. IL-10 polymorphisms -1082, -592 and +3367 and inferred haplotypes were determined.

Results

High serum IL-10 concentrations on consecutive days were associated with ICU admission. IL-10 levels differed significantly between causative pathogens of CAP. Younger patients (age ≤ 50) homozygous for the AAG haplotype werd at risk for CAP compared to healthy controls (OR 4.9, CI 2.0-12.1). Haplotype did not influence the clinical course of CAP.

Conclusion

Serum IL-10 concentrations are associated with clinical course of CAP and the IL-10 AAG haplotype increases susceptibility to CAP.

Introduction

Community-acquired pneumonia (CAP) is a major cause of morbidity requiring hospitalization in approximately 20 percent of all episodes. It is the leading cause of community-acquired infection requiring intensive care unit (ICU) admission, and has an overall mortality of 8.7% [1]. CAP may be caused by many different micro-organisms. Most identified micro-organisms are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Mycoplasma pneumoniae* [2]. In the last few years the role of cytokines in susceptibility to and clinical course of CAP has been investigated [3-6]. The host cytokine response in pneumonia is, to a large extent, compartmentalised to the affected lung, but inflammatory cytokines have also been detected in peripheral blood [5, 7]. Interleukin-10 (IL-10) is an anti-inflammatory cytokine and plays an important role in the innate immune response against viral and bacterial pathogens.

The interindividual variation in the magnitude of the IL-10 response during CAP was recently shown to be dependent of genetic background [6]. Single nucleotide polymorphisms (SNPs) have been associated with different IL-10 production levels [8-10]. For example, the IL-10 -1082 G genotype is associated with increased IL-10 release and seems to be a risk factor for septic shock in pneumococcal infection [11].

Downregulation of pro-inflammatory cytokines would in theory decrease inflammation, however IL-10 excess is able to induce immunosuppression in sepsis and increases morbidity and mortality in pneumococcal pneumonia [12, 13]. IL-10 is necessary to counterbalance proinflammatory cytokines, but overexpression might increase pneumonia severity [11, 12]. High concentrations of IL-10 on admission are associated with nonsurvivors of severe sepsis due to CAP [14]. Recent studies have shown that non-survivors of sepsis had persistently high concentrations of IL-10, while in survivors the IL-10 concentrations decreased over time [15].

Despite this information, the relationship between IL-10 and clinical outcome in CAP remains unclear and the effect of micro-organism on IL-10 concentration in CAP is also not well known. The aim of this study was to evaluate serum IL-10 concentrations during clinical course of CAP and to correlate these with severity of disease and causative micro-organism. Three individual IL-10 SNPs (IL-10 -592 C/A, IL-10 -1082 G/A and IL-10 +3367 G/A) and inferred haplotypes were determined and tested for associations with susceptibility to, and clinical course of CAP.

Methods

Patients and controls

From October 2004 until August 2006, patients with confirmed pneumonia admitted to the emergency department (ED) of the St. Antonius Hospital Nieuwegein were included into this study. Pneumonia was defined as an infiltrate on chest X-ray in combination with at least two of the following criteria: cough, sputum production, temperature >38°C or <35°C, ausculatory findings consistent with pneumonia, C-reactive protein (CRP) >15 mg/l or leucocytosis or –penia (white blood count $>10x10^9$/L, $<4x10^9$/L respectively or >10% rods in leukocyte differentiation). Patients, with defined

immunodeficiencies (a known congenital or acquired immunodeficiency, chemotherapy less than 6 weeks ago, corticosteroids in the last 6 weeks (prednisone equivalent >20 mg/daily for more than 3 days), immunosuppressive medication in the last 6 weeks) or haematological malignancies were excluded. At inclusion clinical and laboratory parameters were recorded [16]. Blood was collected and stored to determine cytokine profile by multiplex immunoassay. Mortality, ICU admission, length of hospital stay and causative agent were prospectively assessed. Criteria for ICU admission were hemodynamic instability or respiratory distress. Written informed consent was obtained from all patients. The study was approved by the institutional Medical Ethical Committee. The control group for the genetic analysis comprised 313 (118 males/195 females) healthy, unrelated employees of the St. Antonius Hospital.

Pathogen identification

For all patients at least two sets of blood cultures and sputum samples (if available) were cultured. Only representative sputum samples were used for bacterial culture. Urine samples were taken for antigen testing of *Legionella pneumophila* serogroup 1 (Binax Legionella urine antigen test) and *S. pneumoniae* cell wall antigen (Binax Streptococcus pneumonia test) [17, 18]. Polymerase chain reactions (Taqman real-time PCR) was performed in sputum to detect *Legionella pneumophila, Mycoplasma pneumoniae* and *Chlamydophila* species [19]. Serological testing (complement fixation) was performed for the presence of antibodies to *Mycoplasma pneumoniae, Coxiella burnetii* or respiratory viruses (influenza A and B, parainfluenza viruses 1, 2 and 3, adenovirus and respiratory syncytial virus) in paired samples taken on day 1 and at least 8 days later. IgG and IgM antibodies to *Legionella pneumophila* were determined on day 1 and 30 by ELISA. For viral cultures (influenza viruses and herpes simplex virus type 1), pharyngeal samples were taken [20].

Study design

We measured venous circulating concentrations of IL-10 on day 1 (day of ED presentation). Subsequent samples were drawn at 8 AM on day 2, 3, 5, 10 and at a control visit at least 30 days after admission. Each blood sample was drawn into pyrogen-free vials and within 2 hours the serum was separated by centrifugation and frozen at -80 °C. The concentration of IL-10 was determined by multiplex immunoassay using a 10-plex human cytokine kit from BioRad Laboratories (Hercules, CA, USA) [21, 22]. The assay was performed according to the manufacturer's instruction and run and analyzed on a Bio-Plex 100 Suspension Array System.

DNA isolation and genotyping

A 200 µl whole blood sample was used to extract DNA with the MagNA Pure LC DNA isolation kit I (Roche) for genotyping of IL-10 -592 C/A (RS1800872), IL-10 -1082 G/A (RS1800896) and IL-10 +3367 G/A (RS3024495). Genotyping was performed on an Applied Biosystems 7500 Fast Real-time PCR system using the TaqMan® technique with customized primers and probes designed by Applied Biosystems and using the manufacturer's instructions. Individual haplotypes were inferred using PHASEv2 software and analyzed if frequencies exceeded a 5% threshold [23].

Statistical analysis

All statistical analyses were performed using statistical software (SPSS version 13.0 for Windows, Chicago). Genotype frequencies of CAP and control patients were tested for conformity to Hardy-

Weinberg equilibrium using the χ^2 test between observed and expected numbers. Associations between IL-10 concentrations, causing pathogen and clinical course were examined using the Mann Whitney U test. Correlations were tested using the Spearman's test.

Results

Baseline characteristics and main outcome parameters

In total 225 patients were initially enrolled in this study. In 201 cases a new infiltrate on X-ray was confirmed by an experienced radiologist. These patients were used for further analysis. We obtained blood for the assessment of cytokine concentrations at the emergency department at presentation from 144 of the 201 enrolled patients. DNA was isolated from 200 of the 201 patients with CAP. Baseline characteristics are shown in Table 1. A definite etiological diagnosis was obtained for 128/201 cases (64%). *Streptococcus pneumoniae* was identified as the most frequent causative agent, followed by *Haemophilus influenzae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* were both found in 9 patients. In 36 patients another causative pathogen was found. During hospital stay, 10 patients died (5%) and 21 patients (10%) were transferred to the intensive care unit because of worsening of disease. Median length of hospital stay was 11 days (interquartile range (IQR): 8-16).

IL-10 concentrations during clinical course of CAP

IL-10 concentrations differed widely in our patients with CAP, from nearly undetectable levels to more than 300 pg/ml. We studied the potential association between IL-10 concentration and clinical course as well as the causative agent. The median IL-10 concentrations on day of admission were similar in patients surviving CAP and patients who did not survive CAP (Figure 1). On consecutive days of sampling IL-10 concentrations remained high in non-survivors, but decreased in survivors, reaching the level of statistically significance on day 10 of hospitalization ($p=0.027$). Analysis of IL-10 concentrations in patients with CAP who needed ICU admission also differed significantly from patients with CAP staying at the ward (Figure 2). Patients who were admitted to the ICU at any time during hospital stay had significant higher median IL-10 levels on day 1 ($p=0.016$), 2 ($p=0.001$), 5 ($p=0.02$) and 10 ($p=0.038$). IL-10 serum levels at control visit also differed significantly ($p=0.026$) between surviving patients who needed ICU admission during hospitalisation and patient without the need of ICU admission.

Table 1: General characteristics of 201 hospitalized patients with community-acquired pneumonia*

Characteristic		
Median age, y (SD)		63.7 ±17
Sex, male		124 (62)
Comorbidity	Diabetes Mellitus	35 (17)
	CVA	17 (9)
	COPD	63 (31)
	Hypertension	38 (19)
Pneumonia Severity Index#:	Low I	30 (15)
	Low II	34 (17)
	Low III	53 (26)
	Moderate IV	56 (28)
	High V	28 (14)
ICU admission		21 (10)
Mortality		10 (5)
Microbiological species:	*Streptococcus pneumoniae*	60 (30)
	Haemophilus influenzae	14 (7)
	Legionella pneumophila	9 (4)
	Mycoplasma pneumoniae	9 (4)
	other	36 (18)
	unknown	73 (36)
Length of hospital stay (median, IQR), days		11 (8-16)

* Data represent the number (percentage) of observations unless otherwise indicated

\# Pneumonia Severity Index based on Fine et al.[16].

In Table 2 IL-10 concentrations on day 1 are compared to day 1 CRP levels. As shown in the previous section IL-10 concentrations on day 1 differed between patients who needed ICU at any time during admission and patients who stayed at the ward (p=0.016). This difference was not seen for CRP levels on day 1. Neither IL-10, nor CRP was a predictor for survival. IL-10 level was statistically significant correlated with length of hospital stay (r_s 0.330; p<0.000), in contrast with CRP levels on day 1 (r_s 0.103; NS). IL-10 as well as CRP were not correlated with Fine score.

Table 2: IL-10 and CRP levels at admission in survivors and non-survivors and in patients with and without the ICU admission

	Survival		ICU admission	
	Yes mean (IQR)	**No** mean (IQR)	**Yes** mean (IQR)	**No** mean (IQR)
IL-10 day 1 (pg/ml)	2.87 (1.3-7.5)	2.64 (1.0-37.5)	5.41* (3.1-15.3)	2.37* (1.2-7.4)
CRP day 1 (mg/l)	190 (94-310)	212 (127-284)	261 (98-388)	187 (95-302)

* p=0.016

IL-10 concentrations in relation to causative pathogen

At presentation, patients with pneumonia due to *Streptococcus pneumoniae* had statistically significant higher IL-10 levels than those with non-pneumococcal pneumonia (median 5.78 pg/ml; IQR 1.7-13.2, p=0.015) (Figure 3). In contrast, in patients with CAP due to *Mycoplasma pneumoniae,* IL-10 levels at day of admission were significantly lower at day of admission than in patients with CAP caused by another pathogen (median 1.1 pg/ml; IQR 0.8-1.7, p=0.021). Pneumonia caused by *Streptococcus pneumoniae* is often characterised by high Fine scores. Whether IL-10 levels are correlated with causative agent or Fine score, we corrected for Fine score. IL-10 serum levels on day 1 are not significantly different between patients with CAP caused by *Streptococcus pneumoniae* and CAP caused by *Mycoplasma pneumoniae* when corrected for Fine score.

IL-10 polymorphisms and CAP

Analysis of 200 patients with CAP and 313 controls for the IL-10 -592 C/A, IL-10 -1082 G/A and IL-10 +3367 G/A SNPs was performed (Table 3). Neither group deviated significantly from the Hardy-Weinberg equilibrium (p>0.05). The three SNPs in the IL-10 gene identified four haplotypes (-1082,-592, +3367: ACG, AAG, GCG and GCA). In patients below the age of 50, haplotype AAG was significantly more frequent (p=0.008) and haplotype GCG was significantly less frequent (p=0.011) than in the healthy control patients (Table 4). Haplotype distribution in elderly patients (> 50 years of age) did not differ from the control group. Carriers of two copies of the AAG haplotype were significantly more frequent in CAP patients below the age of 50 (19%) compared to healthy controls (4%) (OR 4.9 CI 2.0-12.1, p=0.000) and compared to patient with CAP > 50 years of age (3%) (OR 6.8 CI 2.1-21.4, p=0.000). There was no association between IL-10 SNPs or haplotypes and parameters of the clinical course of CAP: mortality, ICU admission or length of hospital stay.

Table 3: Genotype frequencies in patients with CAP and healthy controls.
Data are presented as number (percentage)

Genotype		CAP n=200	CAP age <50 n=48	Healthy controls n=313
IL 10 -592 C/A	CC	115 (57)	21 (44)	173 (55)
	CA	70 (35)	18 (38)	126 (40)
	AA	15 (8)	9 (19)	14 (5)
IL 10 -1082 G/A	GG	54 (27)	9 (19)	74 (24)
	AG	90 (45)	16 (33)	170 (54)
	AA	56 (28)	23 (48)	69 (22)
IL 10+3367 G/A	GG	134 (67)	35 (73)	225 (72)
	AG	63 (31)	13 (27)	77 (25)
	AA	3 (2)	0 (0)	11 (3)

Table 4: Haplotype frequencies in patients with CAP and healthy controls

Haplotype	CAP n = 200 (%)	CAP (age < 50) n = 48 (%)	Healthy controls n = 313 (%)
ACG	103 (26)	26 (27)	154 (25)
AAG	99 (25)	36 (38)*	154 (25)*
GCG	128 (32)	21 (22)#	219 (35)#
GCA	69 (17)	13 (14)	99 (15)
GAG	1 (0)	0 (0)	0 (0)

CAP patients < 50 years of age and controls * p = 0.008, # p = 0.011

Several IL-10 polymorphisms have been associated with increased IL-10 production in vitro, mainly in IL-10-1082 allele G and IL-10-592 allele C homozygous patients [9, 11, 24].
In patients with the -1082 polymorphism the median IL-10 fell rapidly from day 1 to day 2, but remained slightly elevated throughout the first 10 days. On day 30, IL-10 was back to nearly undetectable levels as in healthy adults. The data do not indicate a statistically significant higher IL-10 production in homogygous -1082 G patients, nor in homozygous -592 C patients. In homozygous +3367 G patients IL-10 levels were significant lower on day 1 (p=0.019), however this group consisted of only 3 patients. Moreover IL-10 levels on the following days did not significantly differ from allele A homozygous patients or heterozygous patients. Furthermore, all the different IL-10 haplotypes showed similar magnitude and kinetics of IL-10 production.

Discussion

In this study we found that serum IL-10 concentrations on admission were associated with ICU admission, length of hospital stay and causative agent of CAP. This effect was not due to genetic differences because neither the haplotypes, nor the individual SNPs (-1082 G/A, -592 C/A and +3367 G/A), did influence the IL-10 serum concentrations. However, susceptibility to CAP indeed was influenced by IL-10 haplotype. The group of CAP patients below the age of 50 years contained significantly more homozygous AAG carriers (19%) compared to healthy patients (4%) and compared to patient with CAP > 50 years of age (3%).

Like other studies reported, we found a relationship between IL-10 concentration and severity of the CAP episode [7, 14]. IL-10 is an anti-inflammatory cytokine and is necesary to counterbalance the proinflammatory cytokines. A balance between pro- and anti-inflammatory cytokines maintains the inflammatory response compartmentalized in the lungs and within the limits of homeostasis. However, overexpression of IL-10 might increase pneumonia severity due to immunosuppression. This was in accordance with our findings that the IL-10 serum concentration was significantly higher in patients who needed ICU admission during hospitalisation. IL-10 serum concentration on admission, as a single biomarker, was shown to be superior to CRP in predicting clinical outcome of CAP,

but the prognostic value is too low to use IL-10 as a single predictor of clinical course in an individual patient. Besides IL-10 and CRP, more (inflammatory) biomarkers could predict clinical outcome, like IL-6 and γ-IFN. Subsequent prospective studies should be aimed to identify the optimal combination of these markers to allow for a better prognostic score system.

We have shown in this study that IL-10 concentrations are related to the causative pathogen. Patients with CAP due to *Streptococcus pneumoniae* had significant higher IL-10 levels, and in patients with CAP due to *Mycoplasma pneumoniae* IL-10 levels were lower compared to all other pathogens. The cell wall of *Streptocoocus pneumoniae* is a potent inducer of inflammation, probably via the activation of complement and the induction of cytokines such as TNF-α and IL-6 [25]. *Legionella pneumophila* and *Mycoplasma pneumoniae* are predominantly intracellular living bacteria and therefore differ in their interference with the immune system. It is known that high IL-10 levels inhibit macrophage activation which would allow survival of these bacteria [26].

IL-10 polymorphisms have been associated with susceptibility to meningococcal disease and rheumatoid arthritis [27-30]. Although in experimental models IL-10 polymorphisms have been associated with susceptibility to specific infections, including *Streptococcus pneumoniae*, the clinical susceptibility to CAP thus far does not appear to be influenced by IL-10 polymorphism [13]. Susceptibility and clinical course of CAP in elderly is to a large degree due to the presence of comorbidities. In younger patients with CAP it is unlikely that the presence of comorbidities would play a role. This is in accordance with the finding that IL-10 haplotypes only in the younger patients appear to differ from healthy controls. We did not observe these differences in the elder patients.

In the last few years several studies suggested that there is a genetic influence on the inflammatory response in severe infectious or auto-immune disease [31-33]. An association is found between patients homozygous for the IL-10-1082 G allele and sepsis severity in pneumococcal infection [11, 24]. Two studies have shown that severity of CAP is significantly associated with this genetic polymorphism of IL-10. The severity of CAP as assessed by SIRS score was found to be associated with the -1082 G allele frequency and the IL-10 haplotype -592C/734G/3367G was found to be associated with increased mortality in critically ill patients with pulmonary sepsis [6, 33] Due to complete linkage disequilibrium between the 734G and the –1082A polymorphisms, we could determine the same haplotypes in our patient group but could not confirm this finding: mortality in patients with at least one ACG haplotype is comparable to that in the non-ACG patients (50 and 50%, respectively). Furthermore, we did not find an association between IL-10 polymorphism (-1082 G/A, -592 C/A and +3367 G/A) and mortality, ICU admission or length of hospital stay. These conflicting results with our study can be explained by differences in patient selection criteria (e.g. only ICU patients studied) as well as clinical endpoints (e.g. SIRS).

The three IL-10 gene polymorphisms tested in this study and inferred haplotypes did not influence the level or course of IL-10 concentration during CAP. The general trend for the IL-10 response was a high concentration at admission and thereafter a general decrease with a small secondary peak at day 10. These data are in accordance with earlier studies in an experimental human endotoxemia model in which no association between IL-10 -1082 promotor polymorphism and IL-10 concentrations was found [8]. Yet, other studies in which IL-10 levels in LPS and *Streptococcus pneumoniae*

stimulated whole blood or periferal blood mononuclear cells (PBMC) ex vivo were measured did find an effect of the -1082 and -592 promotor polymorphism [9, 11, 24, 34] It is questionable whether IL-10 production in vivo will reach these maximum stimulation levels.

The purpose of our study was to validate the clinical usefulness of IL-10 serum measurement during CAP and to identify the influence of IL-10 genotype on CAP. In conclusion, serum IL-10 concentrations, in contrast to CRP levels, were associated with ICU admission, length of hospital stay and causative agent but the prognostic value is to low to use IL-10 as a single predictor of clinical course in an individual patient. Moreover, the genetic susceptibility to CAP in patients below the age of 50 was associated with the AAG haplotype.

Figure 1: Serum IL-10 concentrations in association with mortality in patients with CAP.
Box-and-whiskerplot of the IL-10 data, showing the 2.5, 25, 50, 75 and 97.5% cumulative, relative frequencies (centiles). * p = 0.027

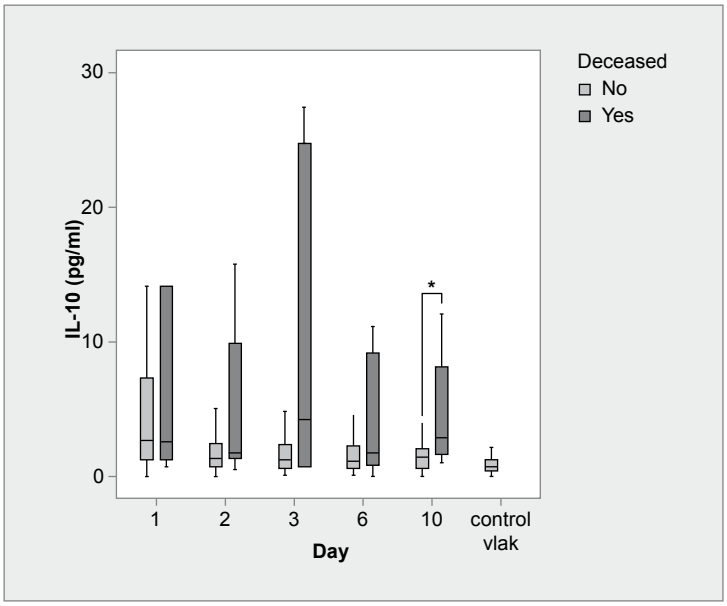


Figure 2: Serum IL-10 concentrations in association with ICU admission in patients with CAP. Box-and-whiskerplot of the IL-10 data, showing the 2.5, 25, 50, 75 and 97.5% cumulative, relative frequencies (centiles). * p < 0.05, ** p < 0.01

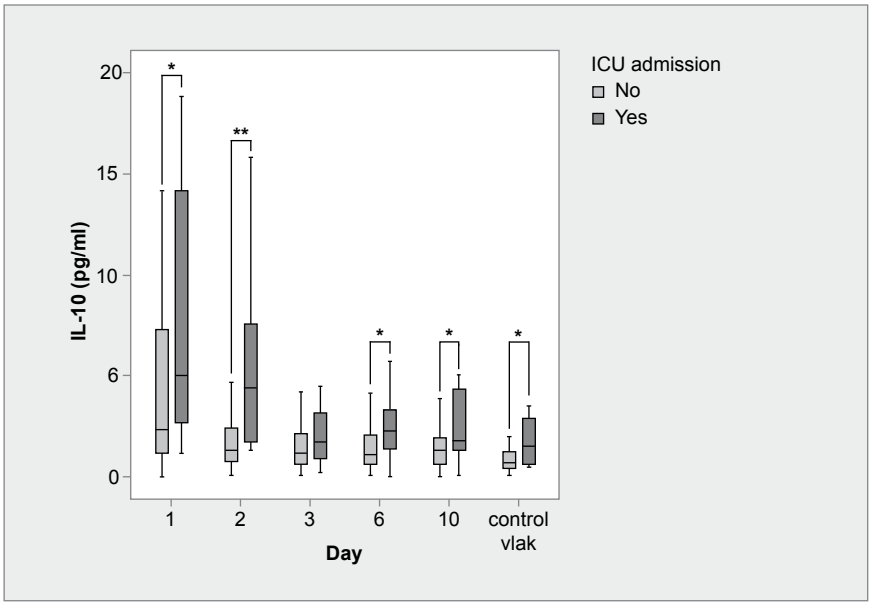
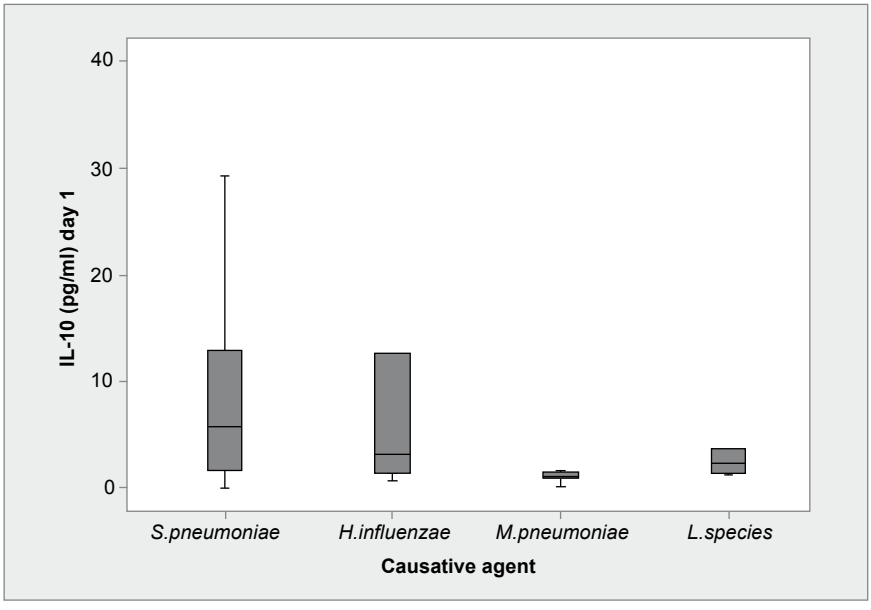


Figure 3: Median serum IL-10 levels on day of hospital admission in patients with CAP caused by either *S. pneumoniae* (n= 44), *H. influenzae* (n=10), *M. pneumoniae* (n=7) or *Legionella* species (n=7). Box-and-whiskerplot of the IL-10 data, showing the 2.5, 25, 50, 75 and 97.5% cumulative, relative frequencies (centiles).



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Part II: Innate immunity

Chapter 9

Toll-like receptor polymorphisms in community-acquired pneumonia

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Abstract

Background

Community-acquired pneumonia (CAP) is caused by *Streptococcus pneumoniae* and *Haemophilus influenzae*. As such, Toll-like receptors (TLRs) are involved in innate immunity against these pathogens, while single nucleotide polymorphisms (SNPs) within TLR genes result in altered functionality of the receptor. The aim of this study was to search for associations between SNPs in the genes of TLR2, 4 and 5 and susceptibility to and the clinical outcome of CAP.

Methods

Two hundred patients with CAP and 311 healthy controls were included in this study. Three TLR-SNPs were identified: TLR2 2477 A>G (Arg753Gly; rs5743708), TLR4 12874 A>G (Asp299Gly; rs4986790) and TLR5 1174 C>T (392>stop; 5744168).

Results

The TLR2 2477 A allele was more frequent in patients with CAP as compared to controls (OR 2.90, CI 1.75-5.11). A higher incidence of the TLR2 2477 A allele was also observed in patients with *Haemophilus influenzae* pneumonia (OR 6.90, CI 2.11-22.62). Prevalence of the TLR5 1174 T allele was significantly higher in patients with pneumococcal pneumonia, as compared to the controls (OR 2.08, CI 1.05-4.12), and was associated with inflammatory markers and invasive disease.

Conclusions

Patients with CAP in general and CAP caused by *Haemophilus influenzae* more frequently carry the TLR2 2477 A allele. The TLR5 1174 T allele is associated with CAP caused by *Streptococcus pneumoniae* and invasive disease.

Introduction

Community-acquired pneumonia (CAP) is the infectious disease that most frequently requires hospitalization in the Netherlands. Common causative pathogens of CAP include *Streptococcus pneumoniae* and *Haemophilus influenzae* [1]. The etiology, clinical course and outcome of CAP depend on various host and pathogen characteristics, such as age, the presence of cardiopulmonary diseases and the causative microorganism. It should be noted that variations in the genetic make-up of the immune system may also play a role [2].

Toll-like receptors (TLRs) are transmembrane receptors that activate an inflammatory response after recognition of pathogen-associated molecular patterns. TLR2 is able to bind bacterial lipoproteins and lipoteichoic acids, while TLR4 binds lipopolysaccharide (LPS) and TLR5 acts as a flagellin receptor [3-9]. The roles of TLRs in pneumonia have been examined in experimental models. TLR2$^{-/-}$ mice intracerebrally infected with *Streptococcus pneumoniae* have more disseminated disease and reduced survival compared to similarly infected wild-type animals. However, a comparable TLR2$^{-/-}$ model of pneumococcal pneumonia showed no differences in outcome between wild-type and knock-out mice [10-12]. Pneumococcal pneumonia in TLR4$^{-/-}$ mice was characterized by a slightly reduced inflammatory response. On the other hand, TLR4$^{-/-}$ mice with Gram-negative pneumonia (including that cause by Legionella) had significantly lower bacterial clearance and survival, as compared to wild-type mice [13-18]. The role of TLR5 has not been studied previously in a mouse model of pneumonia.

Some single nucleotide polymorphisms (SNPs) in the TLR2, 4 and 5 genes have been studied in patients with infectious diseases. The TLR2 2477 A>G (Arg753Gln) SNP is associated with a reduced inflammatory response after infection with *Borrelia burgdorferi* and *Treponema pallidum*, life- threatening bacterial infections, and increased susceptibility to tuberculosis [19-20]. The TLR4 12874 A>G (Asp299Gly) SNP has been linked to a diminished response after contact with LPS, increased risk for septic shock caused by Gram-negative infections, severe clinical course of a viral infection and increased susceptibility to invasive pneumococcal disease in children [21-25]. The TLR5 1174 C>T (392stop) SNP has been found in patients with Legionella infections, probably due to diminished flagellin recognition in the context of a suppressed IL-6 response [26].

Herein, we examined whether TLR2 2477 A>G (Arg753Gln), TLR4 12874 A>G (Asp299Gly) and TLR5 1174 C>T (392stop) SNPs were associated with susceptibility to CAP following infections with various causative micro-organisms or with the clinical course of CAP.

Methods

Patients and controls

Adult patients presenting with CAP at the emergency department of a 600-bed teaching hospital during a period of 22 consecutive months were eligible for participation in this study. Pneumonia was defined by observation of a new infiltrate on chest X-rays, which was confirmed by an experienced radiologist, and two out of six clinical signs of pneumonia (cough, production of sputum, tempera-

ture >38°C or <35° C, white blood count >10x10^9/L or <4x10^9/L, >10% rods in the differential count and C-reactive protein (CRP) >15 mg/l). Patients with a history of recent (<30 days) hospitalization or congenital or acquired immunodeficiency were excluded from this study. Clinical and laboratory data (including interleukin-6 (IL-6) and CRP levels) were collected at presentation and on days 2, 3, 5 and 10 of hospitalization. In addition, a Fine-score was calculated at presentation [27]. Clinical characteristics of this population have been previously described [28]. The control group comprised 311 healthy, unrelated, Dutch Caucasian adults. This study was approved by the local Medical Ethics Committee and informed consent was obtained from each patient.

Pathogen identification

The diagnostic protocol was aimed to identify as many causative pathogens as possible. This protocol has been described and reviewed elsewhere for use in determination of the risk of negative pathogen-identification [28]. Briefly, the protocol consisted of growing cultures of sputum and blood, urine antigen testing for *Streptococcus pneumoniae* and *Legionella pneumophila*, polymerase chain reaction analysis of DNA from the sputum for detection of atypical pathogens (*Chlamydophila pneumoniae/psittaci*, *Legionella pneumophila* and *Mycoplasma pneumoniae*), viral culture of the pharynx (influenzae viruses and Herpes Simplex Virus 1), and serologic testing for respiratory and atypical pathogens (adenovirus, (para-)influenzae viruses, respiratory syncytial virus, *Coxiella burnetii*, *Legionella pneumophila* and *Mycoplasma pneumoniae*).

Genotyping of TLR2 2477 A>G (Arg753Gln), 12874 299A>G (Asp299Gly) and TLR5 1174 C>T (392stop)

Blood was collected from all patients at admission in 10 cc tubes. DNA was extracted from 200-µl whole blood samples for further processing using the MagNA Pure LC DNA Isolation Kit I (Roche Diagnostics, Basel). DNA was genotyped on an Applied Biosystems TaqMan® 7500 Fast Real-time PCR system. For genotyping, customized primers were obtained from Applied Biosystems. The reactions were incubated for 10 min at 95ºC, submitted to 40 cycles of 15 sec at 95ºC and 1 min at 60ºC, and then cooled.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc, Chicago, Illinois). Descriptive values are denoted as the mean and standard deviation or median and range. Hardy-Weinberg equilibrium was calculated for all TLR-SNPs. The significance of differences in SNP frequencies between groups was calculated by Chi-square tests or, in the case of an invalid sample size, Fisher's exact tests. Differences in CRP and IL-6 levels between the different genotypes were calculated by independent-Student t-tests or non-parametric Mann-Whitney tests, depending upon the distribution of the data. Multivariate analysis was performed by logistic regression analysis containing the Fine-score. A $p<0.05$ was regarded as statistically significant.

Results

CAP

Base-line characteristics of all patients are shown in Table 1. In 128 (64%) patients, the causative micro-organism of CAP was identified. The most frequently identified causative micro-organisms were *Streptococcus pneumonia* and *Haemophilus influenzae*. Isolation of DNA failed in one patient, leaving 200 patients for further analysis. Genotype frequencies of the tested TLR-SNPs are shown in Table 2. All TLR-SNPs were in Hardy-Weinberg equilibrium, and carriage of the TLR2 2477 A allele (Arg753Gln and Gln753Gln) was significantly higher in patients (39/183) compared to controls (26/313) (OR 2.99, CI 1.75-5.11; p<0.001). The frequencies of the other tested TLR-SNPs were similar in patients and controls, while there was no significant effect of age or sex on the frequencies of the tested TLR-SNPs.

Table 1: Base-line characteristics of 201 patients with community-acquired pneumonia

Patient characteristics	
Male, n (%)	124 (62)
Age, mean (SD)	63 (17)
Causative micro-organisms, n (%)	
Streptococcus pneumoniae	60 (30)
Haemophilus influenzae	14 (7)
Legionella pneumophila	9 (5)
Mycoplasma pneumoniae	9 (5)
Staphylococcus aureus	6 (3)
Other Gram-negative bacteria[1]	9 (5)
Other atypical bacteria[2]	3 (2)
Other Gram-positive bacteria[3]	2 (1)
Viruses	16 (8)
Unidentified	73 (36)

[1] *Klebsiella pneumoniae* (n = 4), *Pseudomonas aeruginosa* (n = 2), other (n = 3)

[2] *Chlamydophila psittaci/pneumoniae* and *Coxiella burnetii*

[3] Other streptococcal species

The relationship between the TLR-SNPs and clinical outcome is given in Table 3. No differences were found for mortality, ICU admission or length of hospitalization for the tested TLR2 and TLR4 SNPs. Carriage of the TLR5 1174 T allele (392stop) was significantly higher in patients who suffered from invasive disease (bacteraemia): 11/165 (7%) versus 7/35 (20%) (OR 3.69, CI 1.30-10.45, p<0.05). This finding remained statistically significant in multivariate analysis containing the Toll-like receptor genotypes and the Fine-score. A non-significant tendency was found for mortality of patients carrying the TLR5 1174 T allele (p=0.08). Levels of CRP and IL-6, which are both biomarkers of an inflammatory response, were compared among the tested TLR genotypes. Patients carrying the TLR5 1174 T allele had significantly higher levels of CRP and IL-6 (median 311 versus 114 pg/ml;

p<0.001, respectively) at presentation as compared to patients without the T allele (Figure 1). No differences were found in CRP and IL-6 levels among the other tested TLR-SNPs.

Table 2: Frequencies of TLR polymorphisms in CAP in general, CAP caused by the main causative micro-organisms and controls, n (%)

Genotype		Controls (n=313)	CAP (n=200)[#]	*Streptococcus pneumoniae* (n=60)	*Haemophilus influenzae* (n=14)	*Legionella pneumophila* (n=9)	*Mycoplasma pneumoniae* (n=9)
TLR2 2477A/G	GG	287 (92)	144 (79)[1]	50 (91)	8 (62)[3]	7 (78)	6 (75)
(Arg753Gln)	AG	26 (8)	38 (21)[1]	5 (9)	5 (39)[3]	2 (22)	2 (25)
	AA	0	1 (1)[1]	0	0[3]	0	0
TLR4 12874A/G	AA	280 (89)	171 (86)	52 (87)	12 (86)	5 (56)[4]	8 (89)
(Asp299Gly)	AG	32 (10)	27 (14)	8 (13)	2 (14)	4 (44)[4]	1 (11)
	GG	1 (0)	2 (1)	0	0	0[4]	0
TLR5 1174C/T	CC	273 (87)	165 (83)	46 (77)[2]	13 (93)	6 (66)	8 (89)
(392stop)	CT	39 (12)	34 (17)	13 (22)[2]	1 (7)	3 (33)	1 (11)
	TT	1 (0)	1 (1)	1 (2)[2]	0	0	0

[#] In 17 patients, TLR2 2477A/G-SNP analysis failed, leaving 183 patients for further analysis.

[1] A allele carriage: OR 2.99, CI 1.75-5.11; p = 0.000 (χ^2-test)

[2] T allele carriage: OR 2.08, CI 1.05-4.12; p = 0.03 (χ^2-test)

[3] A allele carriage: OR 6.90, CI 2.11-22.62; p = 0.004 (χ^2-test)

[4] G allele carriage: OR 6.79, CI 1.74-26.54; p = 0.01 (Fisher's exact test).

CAP caused by *Streptococcus pneumoniae*

The TLR5 1174 T allele was more frequent in patients with CAP caused by *Streptococcus pneumoniae* (14/60), as compared to controls (40/313; OR 2.08, CI 1.05-4.12; p<0.05) (Table 2). There were no differences in the frequencies of the other tested TLR-SNPs between patients with pneumococcal pneumonia and controls (Table 2). The TLR5 1174 T allele was associated with higher mortality (3/11 versus 0/46; OR 1.27, CI 0.97-7.67; p<0.05) and ICU admission (4/14 versus 2/46; OR 8.80, CI 1.41-54.91; p<0.05) as compared to patients that did not carry the T allele. Furthermore, there was a trend for more invasive diseases in these patients (7/14 in T allele carriers versus 10/42 in non-T allele carriers; cultures were missing for four patients). The length of hospital stay was borderline significant by one-way ANOVA (p=0.049), but not this difference did not reach statistical significance in the independent sample t-tests between carriage of the T allele and non-T allele: mean duration of hospital stay of patients carrying the T allele was 27 days (SD 37) versus 13 days (SD 10) in patients without T allele carriage. The tested TLR2 and TLR4 SNPs had no effect on the clinical outcome of pneumococcal pneumonia. In addition to susceptibility, carriage of the TLR5 1174 T allele is associated with clinical outcome of pneumococcal pneumonia.

Table 3: Frequency of TLR polymorphisms in non-survivors, patients admitted to the ICU and patients with invasive disease, n (%)

Genotype		Non- survivor		ICU admission		Bacteremia*		Hospital stay (days) Median (min-max)
		Positive (n=10)	Negative (n=190)	Positive (n=21)	Negative (n=179)	Positive (n=18)	Negative (n=163)	
TLR2 2477A/G								
(Arg753Gln)[#]	GG (n=144)	6 (67)	138 (79)	17 (85)	127 (78)	16 (94)	113 (75)	11 (5-153)
	AG (n=38)	3 (33)	35 (20)	3 (15)	35 (20)	1 (6)	36 (24)	12 (5-48)
	AA (n=1)	0	1 (1)	0	1 (1)	0	1 (1)	-
TLR4 12874A/G								
(Asp299Gly)	AA (n=171)	9 (90)	162 (85)	19 (90)	152 (85)	15 (83)	139 (85)	11 (4-153)
	AG (n=27)	1 (10)	26 (14)	2 (10)	25 (13)	3 (17)	22 (13)	9 (3-47)
	GG (n=2)	0	2 (1)	0	2 (1)	0	2 (1)	11 (8-13)
TLR5 1174C/T								
(392stop)	CC (n=165)	6 (60)	159 (84)	15 (71)	150 (84)	11 (61)[1]	139 (85)	10 (3-69)
	CT (n=34)	4 (40)	30 (16)	6 (29)	28 (16)	6 (33)[1]	24 (15)	14 (5-153)
	TT (n=1)	0	1 (1)	0	1 (1)	1 (6)[1]	0	-

[#] In 17 patients, TLR2 2477A/G-SNP analysis failed, leaving 183 patients for further analysis

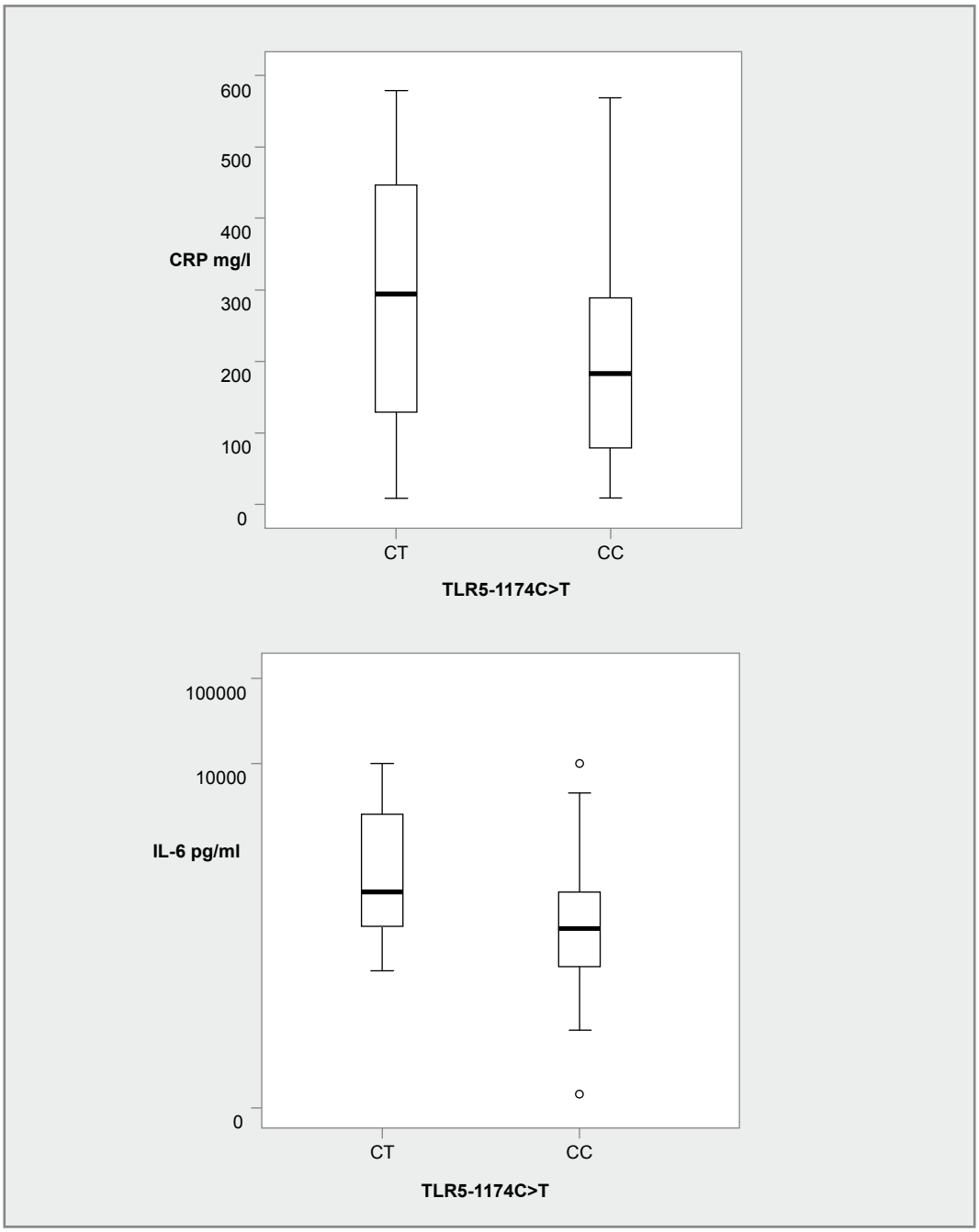
* in 19 patients, no cultures of blood were taken

[1] T allele carriage: OR 3.69, CI 1.30-10.45, p = 0.02 (χ^2-test).

CAP caused by *Haemophilus influenzae, Legionella pneumophila* and *Mycoplasma pneumoniae*

Carriage of the TLR2 2477 A allele was more frequently found in patients with *Haemophilus influenzae* CAP (5/13; TLR2 2477 genotyping failed in one patient) than in the controls (26/313; OR 6.90, CI 2.11-22.62; p<0.005). The frequencies of the genotypes of the TLR4 and TLR5-SNPs did not differ between patients with *Haemophilus influenzae* pneumonia and controls (Table 2). SNPs of TLR4 and 5 had no detectable relationship with clinical course of CAP caused by *Haemophilus influenzae*. Carriage of the TLR4 12874 G allele was significantly more frequent in patients with *Legionella* pneumonia as compared to controls (4/9 versus 33/313; OR 6.79, CI 1.74-26.54; p<0.05). In patients with CAP caused by *Mycoplasma pneumonia*, carriage of the TLR2 12874 A allele was slightly more prevalent compared to controls (25% versus 8%; ns) (Table 2).

Figure 1: C-reactive protein and IL-6 (log scale) serum concentrations at presentation in patients with the TLR5 392CC and CT genotype (median, IQR and min-max).



The one patient with the TLR5 1174TT genotype had a CRP serum level of 489 mg/l and an IL-6 serum level of 3,718 pg/ml. Differences in CRP and IL-6 serum levels between the TLR5 392CC and CT genotype were statistically significant (CRP t-test p = 0.033, IL-6 Mann-Whitney p = 0.000).

Discussion

We report an association between genotypes that encode for reduced functionality of TLR2 and TLR5 and susceptibility to CAP. Furthermore, the TLR5 SNP described herein is observed more frequently in patients with severe CAP that is characterized by bacteraemia. Carriage of the TLR5 1174 T allele (392stop), which encodes for dysfunctional TLR5, is linked to increased susceptibility to pneumococcal CAP. Gram-negative pneumonia (mostly *Haemophilus influenzae*) is more often diagnosed in patients with SNPs that that result in defective TLR2 (Gln753Gln or Arg753Gln). Despite a low number of patients, the SNPs in TLR4 and TLR5 are found to be associated with CAP caused by *Legionella pneumophila*.

TLR5 is a flagellin recognition receptor. The TLR5 1174 T allele (392stop) encodes for a partially dysfunctional receptor that exhibits decreased binding to flagellin [4, 26, 29-30]. We observed a correlation between the TLR5-SNP and *Streptococcus pneumoniae* CAP in a group of patients with no other flagellin-containing pathogens (such as *Legionella pneumophila*). TLR5 1174 T allele carriage was also associated with a poor clinical outcome in terms of invasive disease, which is mainly observed in patients with pneumococcal pneumonia. It is possible that TLR5 recognizes not only flagellin, but also yet to be determined structures of *Streptococcus pneumoniae*. As such, the interaction between TLR5 and *Streptococcus pneumoniae* is of interest for further research. In contrast to TLR5, there are experimental data in support of a TLR2 and TLR4 in *Streptococcus pneumoniae* infections. In mice, TLR2 plays an important role in *Streptococcus pneumoniae* meningitis, but not in pneumococcal pneumonia [10-12]. In $TLR4^{-/-}$ mice, survival was significantly reduced after intranasal injection with *Streptococcus pneumoniae* [15]. Little is known of the role of TLRs in human *Streptococcus pneumoniae* disease. Our study confirms previous work that the TLR2 2477A>G SNP and the TLR4 12874A>G SNP are not associated with susceptibility to or outcome of invasive pneumococcal disease in a mixed population of children and adults (73% pneumonia) [31].

Haemophilus influenzae is the second most frequent causative micro-organism of CAP; this finding was observed in our study population as well as in previous populations. Patients with *Haemophilus influenzae* had a higher frequency of carriage of the TLR2 2477 A allele; however, there is no experimental model for evaluation of the role of TLR in *Haemophilus* pneumonia. MyD88 (the main TLR signaling intermediate) knock-out mice infected with another Gram-negative micro-organism (*Pseudomonas aeruginosa*) had a decreased survival [32]. Similar results have been found in $TLR4^{-/-}$ mice infected with *Klebsiella pneumoniae* [15]. TLR2 is capable of recognizing bacterial lipoproteins and lipoteichoic acid on the outer surface of the membranes of Gram-negative bacteria. This finding may explain our observation of the increased incidence of an SNP that results in reduced efficacy of TLR2 in patients with *Haemophilus influenzae* pneumonia. Carriage of the TLR2 A allele was also higher in a small group of patients with CAP caused by other Gram-negative bacteria (e.g., *Klebsiella pneumoniae*); these findings should be confirmed in a larger cohort of patients.

Legionella pneumophila is a Gram-negative intracellular organism with a flagellin that is recognized by TLR2, TLR4 and TLR5 [4, 26, 29-30]. In experimental models, bacterial load is increased in $TLR2^{-/-}$, but not in $TLR4^{-/-}$ mice relative to wild-type mice [13, 16]. The intracellular effector protein MyD88 is essential for the survival following Legionella infection [16]. The TLR4 12874 AA genotype

(wild-type) is reported to be a risk factor for Legionnaires disease and the TLR5 1174 T allele (stop) is known as a risk factor for infection with *Legionella pneumophila*; both of these genotypes result in diminished recognition of flagellin relative to other genotypes [26, 33]. We observed a higher frequency of the TLR5 SNP in our small population of patients with Legionella pneumonia. However, given the higher frequency of the TLR4 12874 G allele, our study population may be too small to draw any firm conclusions. It is also possible that the role of the TLR4 12874 A>G SNP depends on the serotype or clinical course. While the role of TLR in CAP caused by *Mycoplasma pneumoniae* is unknown, we found a higher frequency of partially dysfunctional TLR4 receptors (carriage of the TLR4 12874 G allele), which suggests that this receptor might play a role in Mycoplasma-associated diseases.
The main limitation of this study is the small sample size of the group of patients with CAP caused by micro-organisms other than *Streptococcus pneumoniae*, on which the sample-size of our study was powered. Differences in genotypes of patients with Gram-negative and atypical pneumonia did not reach statistical significance and should, therefore, be interpreted with caution.

In conclusion, genetic variability in TLRs plays a role in susceptibility to CAP caused by *Streptococcus pneumoniae* (TLR5) and *Haemophilus influenzae* (TLR2). Differences among the genotypes of some TLR SNPs were observed between patients with *Legionella pneumophila* (TLR2, TLR4, TLR5) and *Mycoplasma pneumoniae* (TLR2) pneumoniae, and healthy controls. Patients with genotypes encoding for less functional TLR5 proteins have a poor outcome in terms of invasive disease, which is reflected by an increased inflammatory response.

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Part II: Innate immunity

Chapter 10

The Fcγ receptor II-R/R131 genotype is associated with with severe sepsis in community-acquired pneumonia

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Abstract

Background

Community-acquired pneumonia (CAP) can be caused by a variety of micro-organisms, but is most frequently associated with *Streptococcus pneumoniae* and Gram-negative bacteria like *Haemophilus influenzae*. Encapsulated bacteria are able to escape phagocytosis unless they are bound by IgG2 subclass antibodies. These antibodies interact with Fcγ-receptor IIa (Fcγ-RIIa), thereby facilitating opsonophagocytosis of the encapsulated bacteria. We studied the relationship between the Fcγ-RIIa R/H131 polymorphism and the clinical course of CAP and pathogen-specific susceptibility.

Methods

The Fcγ-RIIa genotype R/H131 was determined in 200 patients with CAP and in 313 healthy controls by PCR and was correlated with clinical course, laboratory parameters, and the causative micro-organism.

Results

The Fcγ-RIIa-R/R131 genotype was more frequent in patients with severe sepsis (OR 2.55; CI 1.30-5.00; $p<0.01$). The majority of patients in this group suffered from invasive pneumococcal disease. The duration of hospital stay was longer in patients with the Fcγ-RIIa-R/R131 genotype. Fcγ-RIIa genotypes were not associated with an increased risk of CAP in general; however, the Fcγ-RIIa-R/R131 genotype was more frequent in patients with CAP caused by *Haemophilus influenzae* compared to controls (OR 3.03; CI1.04-9.09; $p<0.05$).

Conclusion

The Fcγ-RIIa-R/R131 genotype is associated with CAP severity and is more frequent in CAP caused by *Haemophilus influenzae*.

Introduction

Despite advances in diagnostic methods and antibiotic therapy, community-acquired pneumonia (CAP) is still a major cause of morbidity and mortality worldwide. Pneumonia accounts for approximately 10% of total mortality in the Netherlands [1]. *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila,* and *Mycoplasma pneumoniae* are the pathogens most frequently identified in CAP [2, 3].

Both *Streptococcus pneumoniae* and *Haemophilus influenzae* are surrounded by a polysaccharide capsule that protects the bacteria from phagocytosis. However, if IgG2 subclass antibodies recognize and bind capsular polysaccharide antigens, opsonophagocytosis can take place [4-6]. The only receptor able to interact efficiently with IgG2 antibodies is Fcγ receptor IIa (CD32). This receptor is expressed on a variety of cells of the immune system including lymphocytes, macrophages, and polymorphonuclear leukocytes (PMN). The binding of immune complexes by Fcγ receptor IIa (Fcγ-RIIa) of PMNs can induce degranulation and phagocytosis [7, 8].

Due to a single nucleotide polymorphism (guanine (G) into adenine (A)), two co-dominant Fcγ-RIIa allotypes exist with different binding capacities for IgG2. The G to A point mutation at base 494 results in the substitution of arginine (R) by histidine (H) at residue 131 of the protein. Fcγ-RIIa-H131 has a high affinity for IgG2, whereas Fcγ-RIIa-R131 displays a low affinity for IgG2 [7-10]. As a result, phagocytosis of IgG2-opsonized bacteria by homozygous Fcγ-RIIa-R/R131 PMNL is less efficient than phagocytosis by homozygous Fcγ-IIa-H/H131 PMNs. Heterozygous Fcγ-IIa-H/R131 receptors show intermediate phagocytic capacity [11].

Several studies have reported an association between the Fcγ-RIIa polymorphism and meningococcal disease and periodontitis. The association between the Fcγ-RIIa genotype and pneumococcal disease has been examined, however, with conflicting results [7, 9, 12-16]. The association between Fcγ-RIIa and CAP caused by micro-organisms other than *Streptococcus pneumoniae* has not yet been documented. We have investigated the severity of CAP and the causative micro-organisms in relation to the Fcγ-RIIa genotype.

Methods

Patients and controls

Patients diagnosed in the emergency department of the St. Antonius Hospital Nieuwegein with CAP during a 22-month period (October 2004 – August 2006) were included in this study. CAP was defined as an infiltrate on chest X-rays and at least one of the following conditions: cough, sputum production, temperature >38°C or <35°C, ausculatory findings consistent with pneumonia, C-reactive protein >15 mg/l, or white blood count >$10x10^9$/l or <$4x10^9$/l. Exclusion criteria consisted of defined immunodeficiency (congenital or acquired immunodeficiency, chemotherapy in the previous six weeks, corticosteroid administration less than six weeks prior, prednisone equivalent >20 mg/daily for more than three days) and hematological malignancies. The following data were collected at admission: social demographic data, co-morbidities, and pre-hospital treatment. On the

day of admission, the Pneumonia Severity Index score (PSI) was calculated [17]. During hospitalization, patients were closely monitored. The endpoints of the clinical outcome were defined as: length of hospital stay, ICU admission, and occurrence of severe sepsis and death during the hospital stay. Severe sepsis was defined as systemic inflammatory response syndrome in combination with organ failure [18]. The controls consisted of healthy unrelated individuals with the same geographical background. The study protocol was approved by the Institutional Medical Ethical Committee and written informed consent was obtained from all patients.

Receptor genotyping

Blood was taken from all patients and collected in 10 cc containers. DNA was extracted from a 200 µl whole blood sample with the MagNA Pure LC DNA Isolation Kit I of Roche Diagnostics. After extraction, DNA was genotyped on a TaqMan® 7500 Fast Real-time PCR machine from Applied Biosystems. The customized primers and reporters necessary for this genotyping were obtained from Applied Biosytems. The following primer sequences were used: GCTTGTGGGATGGAGAAGGT and CTGGTCAAGGTCACATTCTTCCA. Probes were coded as: CTCCCGTTTGGATCC and TTCTCCCATTTGGATCC. After 10 minutes of incubation at 95ºC, 40 cycles of 15 seconds at 95ºC and 1 minute at 60ºC followed. After a run, the samples were cooled.

Pathogen identification

Pathogens were identified by cultures of sputum and blood, urine antigen testing for *Streptococcus pneumoniae* and *Legionella pneumophila*, polymerase chain reaction of the sputum for *Chlamydophila pneumoniae/psittaci*, *Legionella pneumophila* and *Mycoplasma pneumoniae*, viral culture of the pharynx and serologic testing for respiratory viruses, *Legionella pneumophila*, *Mycoplasma pneumoniae,* and *Coxiella burnetii*.

Statistical analysis

Descripive statistics were performed for all variables. After calculation of the Hardy-Weinberg equilibrium, 2x2 χ^2 tests were used to determine differences in the frequencies of the different Fcγ-RIIa-genotypes between patients and controls and between clinical outcomes within patients. In case of statistically significant results, logistic regression analysis was performed with the significant variable in combination with the PSI Score. Continuous parameters were investigated with independent samples T-tests or Mann-Whitney U tests, depending on the distribution of the data.
Statistical analyses were performed using SPSS14.0 for Windows (Chicago). The significance level was set at $p<0.05$ unless reported otherwise.

Results

Demographics

A total of 201 patients were included in this study. Demographical and clinical characteristics are shown in Table 1. The mean age was 63 years (SD 17) and 124 (62%) subjects were male. In 128 (64%) cases, a causative agent could be identified. In 60 (30%) patients, *Streptococcus pneumoniae* was identified as the causative pathogen of the CAP. *Haemophilus influenzae* was isolated in 14 patients, while *Legionella pneumophila* and *Mycoplasma pneumoniae* were both isolated in

9 patients. During their hospital stay, 21 (10%) patients were admitted to the ICU and 10 (5%) patients died. Control subjects consisted of 314 (118 males) healthy unrelated Caucasians from the same geographical area as the patients.

Table 1: Patient characteristics of 201 hospitalized patients with CAP[§].

Patient characteristics		n (%)
Mean age, years (±SD)		68 ± 17
Sex, male		124 (62)
Pneumonia Severity Index*	I Low	30 (15)
	II Low	34 (17)
	III Low	53 (26)
	IV Moderate	56 (28)
	V High	28 (14)
Clinical outcome	Severe sepsis	54 (27)
	Admission to Intensive Care Unit	21 (10)
	Mortality	10 (5)
Median Length of hospital stay, days (IQR)		11 (8)
Causative micro-organism	*Streptococcus pneumoniae*	60 (30)
	Haemophilus influenzae	14 (7)
	Legionella pneumophila	9 (5)
	Mycoplasma pneumoniae	9 (5)
	Other pathogens	36 (18)
	Unknown	73 (36)

* Pneumonia Severity Index based on Fine et al.[17]

§ Data are presented as number (percentage) unless otherwise indicated.

SD standard deviation

IQR inter quartile range

Fcγ-RIIa genotype and outcome of CAP

In one patient and one control subject, Fcγ-RIIa receptor genotyping failed. The data from the remaining 200 patients and 313 control subjects were used for further analysis. Frequencies of the genotypes were in Hardy-Weinberg equilibrium ($p>0.2$). In patients with the Fcγ-RIIa-R/R131 genotype the frequency of severe sepsis (42%) was significantly higher compared to patients with the Fcγ-RIIa-R/H131 and Fcγ-RIIa-H/H131 genotypes (22%) (OR 2.55; CI 1.30-5.00; $p<0.01$) (Table 2). Logistic regression analysis showed that this finding was independent of the PSI score. The majority of the patients with severe sepsis suffered from CAP caused by an unidentified micro-organism (35%), followed by *Streptococcus pneumoniae* (33%). In patients with bacteremia (all *Streptococcus pneumoniae*) the Fcγ-RIIa R/R131 genotype was non-significantly more frequently present. The frequency of Fcγ-RIIa-R/R131 was non-significantly higher in patients who had been admitted to the ICU during their hospital stay compared to those who had not. In patients with the

Fcγ-RIIa-R/R131 genotype, the duration of their hospital stay was longer compared to patients with the Fcγ-RIIa-H/H131 genotype (p<0.05). This, however, was not independent of the PSI score. Patients with the Fcγ-RIIa-R/R131 genotype, the genotype with lower binding affinity for IgG2, are at risk for a more severe clinical course, which is reflected by a higher risk of severe sepsis and a longer stay in the hospital.

Table 2: Genotype distribution of Fcγ-RIIa genotypes among patients with or without clinical endpoints of CAP[§]

	Severe sepsis		Admission to ICU		Mortality		Duration of hospital stay*
Fcγ-RIIa	Positive	Negative	Positive	Negative	Died	Survived	
RR (CC)	22 (41)[1]	31 (21)	7 (33)	46 (26)	3 (30)	50 (26)	11 (5-153)[2]
RH (CT)	18 (33)[1]	73 (50)	10 (48)	81 (45)	4 (40)	87 (46)	11 (3-69)[2]
HH (TT)	14 (26)[1]	42 (29)	4 (19)	52 (29)	3 (30)	53 (28)	10 (4-42)[2]

ICU: Intensive Care Unit

§ Data are presented as number (percentage) unless otherwise indicated.

* data are presented as median (range) number of days

1 Chi-square RR versus. non-RR: OR 2.55 (CI 1.30-5.00; p<0.01)

2 Mann-Whitney test RR versus non-RR p < 0.05

Fcγ-RIIa genotype and risk of CAP

Table 3 shows the distribution of the Fcγ-RIIa genotypes in patients with CAP and controls. There was no association between the Fcγ-RIIa-R/R131 genotype and susceptibility to CAP in general, nor was there an association with the severity of the CAP at presentation (Fine-class IV and/or V). In order to study the association of the Fcγ-RIIa genotype in patients with CAP caused by specific micro-organisms, we tested the frequencies of genotypes in patients with the most common causative micro-organisms of CAP: *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila,* and *Mycoplasma pneumoniae*. The results are also shown in Table 3. The frequency of the Fcγ-RIIa-R/R131 genotype was significantly higher in patients with CAP caused by *Haemophilus influenzae* (OR 3.03; CI1.04-9.09; p<0.05). There was no association between the Fcγ-RIIa genotypes and CAP caused by *Streptococcus pneumoniae*. The frequency of the R/R131 genotype was lower in patients with CAP caused by *Mycoplasma pneumoniae* compared to controls (OR 0.16; CI 0.04-0.65; p<0.01). For Legionella pneumonia, the distribution of the Fcγ-RIIa genotypes was similar to that of the controls (R/R131 22%, R/H131 44% and H/H131 33%).

Table 3: Distribution of the Fcγ -RIIa genotype in CAP patients and controls and in patients with *Streptococcus pneumoniae* and *Haemophilus influenzae*

Fcγ-RIIa genotype	Controls	CAP	*S. pneumoniae*	*H. influenzae*
RR (CC) (%)	77 (25)	53 (27)	17 (23)	7 (50)[1]
RH (CT) (%)	161 (51)	91 (46)	40 (55)	2 (14)
HH (TT) (%)	75 (24)	56 (28)	16 (22)	5 (36)

[1] Chi-square controls versus *H. influenzae* OR 6.25 (CI 1.54-25; p<0.01)

Discussion

We have shown that the Fcγ-RIIa-R/R131 genotype is associated with more a severe clinical course (severe sepsis, longer stay in hospital) in patients hospitalized for CAP. The Fcγ-RIIa-R/R131 genotype was more frequently found in patients with CAP caused by *Haemophilus influenzae*.

The genetic polymorphism of Fcγ-RIIa has functional implications for the efficacy of phagocytosis of encapsulated bacteria. In vitro research has shown that the uptake of opsonized streptococci [11], staphylococci, *Haemophilus influenzae* type b [19], and *Neisseria* species [20] by PMN from subjects homozygous for Fcγ-RIIa R/R131 is less than the uptake by PMN from subjects homozygous for Fcγ-RIIa H/H131. More recent studies focus on the *in vivo* effects of this polymorphism [9, 14, 15]. It is thought that bacterial clearance in Fcγ-RIIa R/R131 homozygous patients is less effective, thus allowing pathogens to replicate and translocate from the local site of inflammation into the bloodstream. Although such a mechanism is still speculative, our results support this hypothesis. In our study, pneumococcal bacteremia was non-significantly more common in patients with the Fcγ-RIIa R/R131 genotype, a finding that has been reported before in patients with pneumococcal pneumonia [14]. The incidence of severe sepsis, and to a lesser extent bacteremia, was increased in the group of patients with the Fcγ-RIIa R/R131 genotype. Bacteremia was solely caused by *Streptococcus pneumoniae*, which was also the most frequently identified causative micro-organism in cases of severe sepsis. We further demonstrated that the duration of hospital stay was associated with the Fcγ-RIIa genotype, being the lowest in HH homozygous patients, intermediate in RH heterozygous patients, and highest in RR homozygous patients. In conclusion, patients with the Fcγ-RIIa R/R131 genotype may have a more severe clinical course of CAP, possibly due to a higher bacterial load as a result of diminished bacterial clearance (of *Streptococcus pneumoniae*). The role of the Fcγ-RIIa genotype in susceptibility for pneumococcal bacteremia has been studied previously; however, the published results are inconsistent. In children, the Fcγ-RIIa-R/R131 genotype was found more commonly in pneumococcal bacteremia compared to healthy adult controls [15]. Yee et al. found similar results in patients with bacteremic pneumococcal pneumonia, who were more likely to be homozygous for Fcγ-RIIa-R/R131 than patients with non-bacteremic pneumococcal pneumonia or controls [14]. However, Moens et al. reported that the frequency of the Fcγ-RIIa genotypes in patients with invasive pneumococcal disease (pneumococcal bacteremia, meningitis, and arthritis) and controls was similar in an adult population. In 74% of the cases, pneumonia was the focus of infection [9]. In accordance with the latter study, we did not find an association between the Fcγ-RIIa

genotypes and pneumococcal CAP in general, but a possible association between pneumococcal invasive disease and the Fcγ-RIIa-R/R131 genotype.

We did find an association between the Fcγ-RIIa-R/R131 genotype and CAP caused by the Gram-negative bacterium *Haemophilus influenza*, which has not been reported before. It is remarkable that the risk of CAP caused by *Haemophilus influenzae* is influenced by the Fcγ-RIIa genotype, whereas the risk of CAP caused by *Streptococcus pneumoniae* is independent of the Fcγ-RIIa genotype. The reasons for this discrepancy are unknown, but there are several possibilities that might explain these findings. The IgG2 antibody binding to non-polysaccharide surface components such as LPS may differ. Also, host defense against gram negative bacteria might be more dependent upon Fcγ-RIIa-mediated opsonophagocytosis than the host defense against Gram-positive bacteria, such as *Streptococcus pneumoniae*. Clearance of encapsulated bacteria can be established through Fcγ-RIIa mediated opsonophagocytosis, but the impact of this process on development of the infection is highly uncertain and may also depend on the specific pathogen in question. For example, in cystic fibrosis patients, the R allele of Fcγ-RIIa has been associated with an increased risk of acquiring chronic *Pseudomonas aeruginosa* infection [21]. This is in agreement with the hypothesis that clearance of gram negative bacteria is dependent upon Fcγ-RIIa-mediated opsonophagocytosis with IgG2. A comparison between these findings and those in our study is hampered by differences in the study populations with respect to age, ethnicity, and morbidity.

In contrast to the findings in CAP caused by *Haemophilus influenzae*, the frequency of the Fcγ-RIIa H/H131 genotypes was higher in patients with CAP caused by *Mycoplasma pneumoniae*. Although the number of patients in this group is too small to draw conclusions, it is surprising that none of the patients had the R/R131 genotype. *Mycoplasma pneumoniae*, as *Legionella pneumophila*, is an intracellular living pathogen and so might benefit from receptors facilitating phagocytosis, thereby escaping other parts of the immune system. It is clear that the role of the Fcγ-RIIa genotype in susceptibility for CAP caused by specific micro-organisms should be a topic for further research.

The expected Fcγ-RIIa genotype distribution, Fcγ-RIIa-R/R131: Fcγ-IIa-H/R131: Fcγ-IIa-H/H131, in Caucasians is 1:2:1 [22], which is similar to the genotype distribution in our study population. This allows for direct comparison with other studies and/or populations. The strength of this study is the relatively large study population compared to previous studies, although the number of patients with specific pathogens (especially *Mycoplasma pneumoniae*) and severe clinical courses (death or ICU admission) were still too small to draw definite conclusions.

In conclusion, we have demonstrated an association between the Fcγ-RIIa-R/R131 genotype and severe sepsis and longer stay in the hospital for CAP, in general, and a genetic predisposition for CAP caused by *Haemophilus influenzae*.

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Part II: Innate immunity

Chapter 11

Acute-phase responsiveness of mannose-binding lectin in community-acquired pneumonia is highly dependent on *MBL2* genotypes

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Abstract

Background

Mannose-binding lectin (MBL) is a pattern recognition receptor of the complement system and plays an important role in innate immunity. Whether or not MBL acts as an acute-phase response protein in infection has been an issue of extensive debate because MBL responses have shown a large degree of heterogeneity. Single nucleotide polymorphisms (SNPs) in the promoter and exon 1 of the *MBL2* gene can lead to MBL deficiency.

Methods

This study investigated the influence of SNPs in the promoter and exon 1 of the *MBL2* gene on the acute-phase responsiveness of MBL in 143 patients with community-acquired pneumonia.

Results

Acute-phase reactivity was only observed in MBL-sufficient genotypes. In patients with wild-type exon 1 genotype A/A, positive acute-phase responses were associated with the presence of the YA haplotype and negative responses with its absence. Genotypes YA/0 and XA/XA produced equal levels of MBL in convalescence. In the acute phase, however, patients with genotype YA/0 were able to maintain this MBL level, while patients with genotype XA/XA were not. Correlation of MBL and CRP levels in the acute phase of pneumonia also depended on the *MBL2* genotype.

Conclusion

In conclusion, acute-phase responsiveness of MBL was highly dependent on the *MBL2* genotype. These data suggest that heterogeneity in protein responses in the acute phase of disease should always be viewed in the light of possible influences of genetic differences in both structural and regulatory parts of the gene.

Introduction

Mannose-binding lectin is a multimeric pattern recognition protein of innate immunity and activates the lectin pathway of complement. It binds to a variety of microorganisms, including respiratory pathogens like influenza A virus [1], pneumococci, *Haemophilus influenzae* [2] and *Legionella pneumophila* [3]. MBL deficiency has been correlated with increased risk of infection, including repeated respiratory tract infections [4], as early opsonization is compromised.
MBL levels in serum are influenced by single nucleotide polymorphisms (SNPs) in exon 1 and in the promoter region of the *MBL2* gene. Coding SNPs in exon 1 ("0" alleles B, C and D vs. wild-type A allele) lead to non-functional MBL monomers in homozygotes, impairing early complement activation. The X/Y promoter SNP influences MBL serum levels in heterozygotes by controlling transcription of the functional A allele. Genotypes 0/0 and XA/0 display MBL levels <0.2 µg/ml and are considered deficient [5,6].

It has been suggested that MBL acts as an acute-phase protein responding to inflammation, consistent with its role in early infection [7]. However, this has been extensively debated since MBL responses have shown a large degree of heterogeneity. Conflicting results have been described in post-operative patients [8-10], patients with severe infections [11] and patients with community-acquired pneumonia (CAP) [12]. Although some of these studies considered *MBL2* exon 1 polymorphisms in the analysis of MBL acute-phase responsiveness, none of them reported the influence of the X/Y promoter SNP on individual MBL responses.

We determined MBL levels and *MBL2* genotypes in patients with community-acquired pneumonia, and analyzed whether acute-phase responsiveness was associated with the observed genotypes, including the X/Y promoter SNP.

Methods

Patients and controls

All adult patients (>18 years) presenting with CAP in our general 600-bed teaching hospital during the period from October 2004 to August 2006 were included in this study as described before [13]. Patients with a history of recent hospitalization (<30 days) or a congenital or acquired immunodeficiency (including the use of prednisone 20 mg/day for more than three days) were excluded. Pneumonia was defined as a new infiltrate on chest X-ray and the presence of two out of the following six clinical signs of pneumonia: cough, production of sputum, signs of consolidation on respiratory auscultation, temperature >38°C or <35°C, C-reactive protein (CRP) 3 times above the upper limit of normal (5 mg/l), or a leukocyte reaction (leukocytosis (white blood count (WBC) >10 g/l), leukopenia (WBC <4 g/l) or more than 10% rods in the differential count). The chest X-ray was interpreted at presentation in the emergency department by a resident. For this study, it was evaluated the next day by an experienced radiologist who was blinded to the clinical information.
Whole blood samples were taken at day 1 of admission for DNA extraction. Serum samples were drawn at the acute phase (day 1) and during convalescence (day 30 or later) and stored for further analysis. Data on clinical parameters on the day of admission were collected and used to calculate

the pneumonia severity index (i.e., Fine-score) as described before [13,14]. The study was approved by the local Medical Ethics Committee and informed consent was obtained from each patient.

Genotyping of MBL2

Combined MBL X/Y promoter and exon 1 haplotypes of *MBL2* were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay [15] with modifications in a nested PCR protocol [16]. For each sample, two PCR assays specific for the promoter X SNP (forward primer ATT TGT TCT CAC TGC CAC C; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; annealing temperature 63°C; 25 cycles) or Y SNP (forward primer TTT GTT CTC ACT GCC ACG; same reverse primer and PCR conditions) were run. The two PCR products were separately diluted 1:100 in distilled water. *MBL2* exon 1 was amplified from these two dilutions with an extra GC-clamp attached to one primer to meet DGGE requirements (forward primer with 41-bp clamp: CCG CCC GCC GCG CCC CGC GCC CGG CCC GCC GCC CCC GCC CCT CCA TCA CTC CCT CTC CTT CTC; reverse primer: GAG ACA GAA CAG CCC AAC ACG). The amplified DNA was run overnight on a 6% polyacrylamide gel containing a denaturing gradient linearly increasing from 35% to 55% formamide and urea. All *MBL2* exon 1 genotypes could be distinguished by their different patterns of migration. The corresponding *MBL2* X/Y promoter haplotype could be inferred from the presence or absence of a product in the in the two nested PCR assays. Genotypes 0/0 and XA/0 were considered “MBL-deficient”, and genotypes YA/0, XA/XA, XA/YA and YA/YA were considered “MBL-sufficient” [5,6].

MBL ELISA

Serum levels of the multimeric MBL protein were determined using a commercially available ELISA (Sanquin, Amsterdam, the Netherlands). In short, MBL bound to coated mannan was quantified with the use of an anti-MBL antibody recognizing the multimeric form only.

Statistical analysis

Data on *MBL2* genotypes and MBL protein levels in convalescence were analyzed using a receiver operating characteristic (ROC) curve. The cut-off value derived from the ROC-curve analysis was used to assess the MBL level as a predictor of MBL-deficient genotypes 0/0 and XA/0. Serum levels below this cut-off value were considered deficient. The difference in MBL level between the acute and convalescent phase sera was analyzed by genotype to measure the influence of the *MBL2* genotype on acute-phase responsiveness of MBL. A decrease or increase of the MBL level by at least 25% in the acute phase compared to the convalescent phase was considered an acute-phase reaction [11,17]. When patients displayed deficient MBL concentrations in both the acute and convalescent phase, they were classified as not showing acute-phase responsiveness. Genotype groups were compared first by means of univariate analysis. Unpaired continuous variables were analyzed with a Student’s t-test after correction for inequality of variances (based on Levene tests). Normality of the difference between two paired variables was analyzed with use of a Kolmogorov-Smirnov procedure. For paired continuous variables with normally distributed differences, a paired Student’s t-test was used. Paired continuous variables without normally distributed differences were analyzed using a Wilcoxon signed ranks test. Categorical variables were analyzed with a Pearson’s chi-squared test or Fisher’s exact test. To adjust for confounders, multivariate logistical regression models using backward stepwise elimination by likelihood ratio tests were used. Since the Fine-

score incorporates both patient (i.e., age and gender) and clinical characteristics, this score was used as the only extra covariate. To measure the correlation between MBL and CRP levels, the Spearman's rho correlation coefficient was calculated. Data were analyzed with SPSS software version 15.0 (SPSS, Chicago, USA).

Results

For 143 of the 201 included patients, whole blood samples and both acute and convalescent phase serum samples were available for analyzing the dynamics of MBL levels in patients with community-acquired pneumonia. The remaining 58 patients could not be analyzed because no convalescent phase serum sample was available. They did not differ from the remaining study group in MBL levels on day 1 of admission (Student's t-test, p=0.25).

The ROC curve of the data on *MBL2* genotypes and MBL protein levels in convalescence suggested a cut-off value of 0.2 µg/ml for predicting genotypic MBL deficiency. The ROC curve had an area under the curve of 0.961 [95%C.I. 0.925-0.997], and using 0.2 µg/ml as cut-off for MBL deficiency, the sensitivity and specificity were maximized at 87.5% and 91.6%, respectively. Therefore, MBL serum levels below the cut-off value of 0.2 µg/ml were considered deficient.

The *MBL2* genotype groups did not differ in age (ANOVA, p=0.98), sex (χ^2 test, p=0.66), or Fine-score (ANOVA, p=0.50) (Table 1). In overall analysis, the mean MBL level was increased significantly in the acute phase of disease compared to convalescence (Wilcoxon signed ranks test, p<0.001; Table 1). When analyzed by genotype, the mean MBL level was increased significantly only in genotype YA/YA (paired Student's t-test, p<0.01).

Table 1: Patient characteristics and MBL levels in the acute and convalescent phase of CAP by *MBL2* genotype (n = number of patients, M = male, F = female).

MBL2 genotype	n	Age (years ± SD)	Sex (M:F)	Fine-score	MBL (µg/ml) acute phase	MBL (µg/ml) convalescent phase
Total	143	61.1 ± 16.7	1.6:1	81.2 ± 30.6	1.51 ± 1.31 †	1.30 ± 1.10 †
YA/YA	43	60.7 ± 15.6	1.2:1	75.5 ± 27.6	2.73 ± 0.86 ‡	2.23 ± 0.88 ‡
XA/YA	34	61.5 ± 17.1	2.1:1	86.2 ± 36.9	2.11 ± 0.85	1.82 ± 0.77
XA/XA	8	58.5 ± 24.9	1.7:1	76.0 ± 37.6	0.46 ± 0.38	0.66 ± 0.38
YA/0	34	61.9 ± 14.7	2.1:1	87.4 ± 26.0	0.65 ± 0.91	0.62 ± 0.72
XA/0	19	59.6 ± 19.3	1.4:1	78.1 ± 31.5	0.08 ± 0.19	0.11 ± 0.23
0/0	5	65.6 ± 16.5	0.7:1	75.6 ± 21.4	0.00 ± 0.00	0.00 ± 0.00

† $p<0.001$, Wilcoxon signed ranks test

‡ $p<0.001$, paired t-test

The dynamics of MBL levels differed between *MBL2* genotypes (Fig. 1). In genotypes YA/YA and XA/YA, mean serum MBL levels were increased in the acute phase by 30.8% ± 37.5% and 27.7% ± 60.0%, respectively (numbers are the mean of individual relative changes ± SD). In patients with genotype XA/XA, mean MBL levels were decreased (-24.1% ± 43.0%) in the acute phase. Patients with genotype YA/0 showed steady MBL levels (-0.2% ± 42.7%). Of 19 patients with MBL-deficient genotype XA/0, 16 patients had MBL levels <0.2 µg/ml at both the acute and convalescent phase, but 3 patients had MBL levels >0.2 µg/ml at one or both time points. In these three patients MBL levels were decreased in the acute phase of disease (-28.9% ± 43.3%). No MBL dynamics were observed in patients with MBL-deficient genotype 0/0, as all samples showed MBL levels <0.2 µg/ml.

Acute-phase responses of MBL were observed in 55 of 143 (38.5%) patients with CAP, of whom 40 (28.0%) showed a positive acute-phase response of MBL and 15 (10.5%) showed a negative response. Acute-phase responsiveness of MBL differed significantly between the *MBL2* genotypes. Significantly more patients with MBL-sufficient genotypes showed acute-phase responses of MBL than patients with MBL-deficient genotypes (Table 2).

Figure 1: Dynamics of MBL protein levels in CAP according to genotype. The relative change (percent) of the MBL level in the acute phase of disease compared to the convalescent level is plotted, stratified by *MBL2* genotype. The cut-off for positive (+25%) and negative (-25%) acute phase responses are plotted (dotted lines). Positive acute-phase responses were associated with the presence of the YA haplotype and negative responses with its absence.
(◆ = individual patient, ━ = mean)

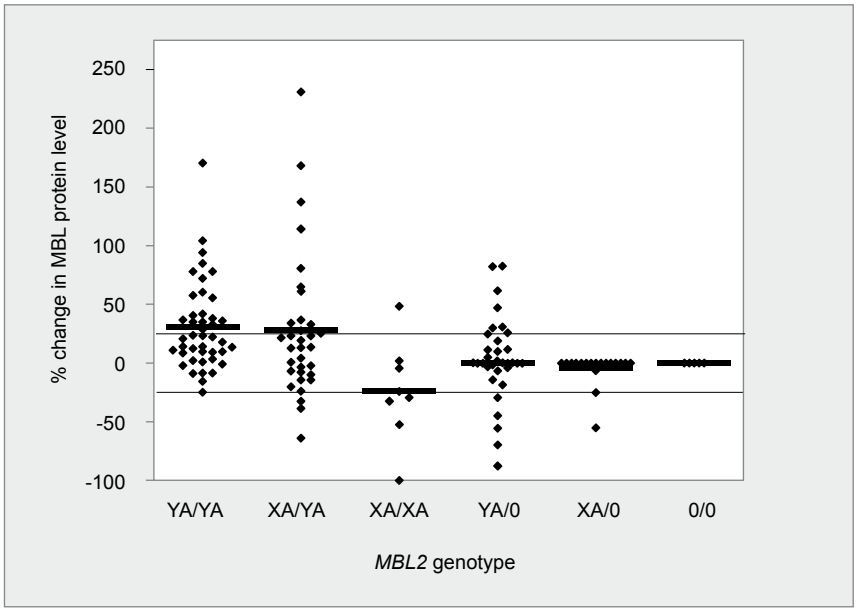

Table 2: Acute-phase responsiveness (APR) of MBL by genotype.

***MBL2* genotype**	**n**	**APR**	**positive APR**	**negative APR**
Total	143	55 (38.5%)	40 (28.0%)	15 (10.5%)
MBL-sufficient	119	53 (44.5%)†	40 (33.6%)	13 (10.9%)
YA/YA	43	20 (46.5%)	19 (44.2%)	1 (2.3%)
XA/YA	34	16 (47.1%)	13 (38.2%)	3 (8.8%)
XA/XA	8	5 (62.5%)	1 (12.5%)	4 (50.0%)‡
YA/0	34	12 (35.3%)	7 (20.6%)	5 (14.7%)‡
MBL-deficient	24	2 (8.3%)†	0 (0%)	2 (8.3%)
XA/0	19	2 (10.5%)	0 (0%)	2 (10.5%)
0/0	5	0 (0%)	0 (0%)	0 (0%)

† p=0.001, χ^2 test, OR 8.8 [95%C.I. 2.0-39.3]

‡ p=0.050, Fisher's exact test

In patients with wild-type exon 1 genotype A/A who displayed acute-phase responsiveness of MBL, positive acute-phase responses were observed significantly more often in patients having at least one wild-type promoter Y allele (32/36 patients with genotype YA/YA or XA/YA vs. 1/5 patients with genotype XA/XA; Fisher's exact test; $p<0.01$). This correlation remained significant when corrected for possible confounding by differences in severity of disease as expressed by the Fine-score in the multivariate logistic regression model (OR 32.0 [95%C.I. 2.9-361.8]). In all A/A patients, negative acute-phase responsiveness was correlated with the absence of the promoter Y allele. MBL showed a negative acute-phase response in 4 of 8 patients (50.0%) with genotype XA/XA, but in only 4 of 77 (5.2%) patients with either genotype XA/YA (3/34 patients) or YA/YA (1/43 patients) (Table 2; Fisher's exact test; $p<0.01$). This correlation also remained significant in the multivariate analysis (OR 0.06 [95%C.I. 0.01-0.30]).

Mean convalescent MBL levels were similar in patients with genotypes XA/XA and YA/0 (Table 1; Student's t-test; $p=0.87$). In the acute phase, however, the mean MBL level was lowered compared to convalescent levels in patients carrying genotype XA/XA, while it remained the same in those with genotype YA/0 (-0.20 ± 0.32 µg/ml vs. 0.04 ± 0.29 µg/ml, respectively; Student's t-test; $p=0.05$). Furthermore, negative acute-phase responses were observed significantly more with genotype XA/XA than with YA/0 (4/8 XA/XA vs. 5/34 YA/0; Fisher's exact test; $p=0.05$).

There was no significant correlation between MBL and CRP at day 1 of admission in overall analysis of all patients (Spearman's rho correlation coefficient -0.049, $p=0.56$). However, in patients with wild-type genotype YA/YA there was a significant correlation between the two parameters (Spearman's rho 0.41, $p=0.01$).

Discussion

Our results show that acute-phase responsiveness of MBL in patients with CAP was highly dependent on the MBL2 genotype. In general, acute-phase responsiveness was observed more frequently in patients with MBL-sufficient genotypes than in patients with MBL-deficient genotypes. In patients with MBL-deficient genotypes, MBL levels were low in both the acute and convalescent phase of disease. In patients with wild-type exon 1 genotype A/A, acute-phase responsiveness of MBL was influenced by the promoter X/Y polymorphism. Positive acute-phase responsiveness was associated with the presence of the YA haplotype, while negative acute-phase responsiveness was associated with its absence. MBL and CRP levels at day 1 were not correlated in the study population as a whole. However, there was a significant correlation between them in patients with wild-type genotype YA/YA.

The current definition of acute-phase responsiveness based on relative changes in the protein level does not take into account whether absolute MBL levels are considered deficient. In this study, we considered patients with MBL levels below 0.2 µg/ml at both the acute and convalescent stage of pneumonia to be MBL-deficient at all time points and therefore classified them as not having an acute-phase response of MBL. The acute-phase response comprises a large number of systemic changes accompanying inflammation that can be distant from the site of inflammation and can

involve many organ systems [17]. The concentration of many plasma proteins is changed during the acute-phase response, including several complement components [17]. Whether MBL exhibits an acute-phase response has been an issue of extensive debate since this was first postulated [7], as MBL responses have shown a large degree of heterogeneity. Monitoring of MBL serum levels after surgery has shown conflicting results [8-10]. When the *MBL2* exon 1 genotype was considered, postoperatively increased MBL levels were found only in patients carrying wild-type exon 1 A/A alleles [18]. In patients with severe infection, no clear acute-phase response was evident from mean MBL levels, even when stratified by exon 1 genotypes (A/A versus A/0 and 0/0) [11]. However, 58.6% of the patients did show an acute-phase response either by increased (31.4%) or decreased (27.3%) MBL levels in the acute phase. It was found also that in community acquired pneumococcal pneumonia, MBL did not act uniformly as an acute-phase reactant in all patients and no correlation with CRP levels was found [12].

Our results show that besides exon 1 SNPs, the promoter X/Y polymorphism can influence individual MBL acute-phase responsiveness, too. The deficient genotypes, XA/0 and 0/0, cannot display an acute-phase response, as MBL activity is absent at all phases. Positive and negative acute-phase responses in all other genotypes are reflected by the balance of upregulating transcription of the wild-type A allele and the consumption of MBL. The promoter X SNP is thought to hamper this upregulation. The influence of the X/Y promoter SNP on the capability of mounting an acute-phase response could be an explanation for the heterogeneity in MBL responses described in the studies above [10-12]. Furthermore, our results show that a significant correlation between MBL and a known acute-phase reactant, CRP, could only be demonstrated when genotypes were taken into account. The effect of the X/Y promoter SNP on acute-phase responsiveness was also reflected in the different responses of genotypes XA/XA and YA/0. Both genotypes displayed comparable intermediate MBL levels in convalescence. In the acute phase, however, patients with genotype YA/0 were able to maintain this MBL level, while patients with genotype XA/XA were not. Therefore, genotype XA/XA should be categorized as only MBL-intermediate and genotype YA/0 as MBL-sufficient, despite equal levels at convalescence. Furthermore, it could be argued that if certain patient groups were to be supplemented with MBL, this supplementation should not be restricted to MBL-deficient genotypes but should include genotype XA/XA as well.

In conclusion, our data show that MBL acute-phase responsiveness is highly dependent on the MBL2 genotype, where the X/Y promoter SNP determines the capability of mounting positive acute-phase responses in individuals with exon 1 wild-type genotype A/A. These data suggest that heterogeneity in protein responses should always be viewed in the light of possible influences of genetic differences in both the structural and the regulatory parts of the gene.

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Part II: Innate immunity

Chapter 12

Mannose-binding lectin genotypes in susceptibility to community-acquired pneumonia

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Abstract

Background

Community-acquired pneumonia (CAP) is most frequently caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, atypical pathogens and respiratory viruses. Susceptibility to CAP can be increased by single nucleotide polymorphisms (SNPs) within the mannose-binding lectin (MBL) gene. We questioned whether MBL polymorphisms are associated with the susceptibility to and outcome of CAP and its most common pathogens.

Methods

All adult patients presenting with CAP in a 23-month period were included in this study. Frequencies of SNPs were determined for the promoter X/Y and the three coding SNPs in exon1 (A/0). Six genotypes were constructed representing patients with sufficient and deficient serum levels of MBL. The results of the patients with CAP were compared with controls.

Results

In 199 patients and 223 controls MBL genotypes were determined. There were no differences in MBL genotype frequencies between patients with CAP in general, pneumonia caused by *Streptococcus pneumoniae* or *Haemophilus influenzae*, and controls. The frequency of sufficient MBL genotypes was non-significantly higher in patients with pneumonia with *Legionella pneumophila* and *Mycoplasma pneumoniae*. In *Legionella pneumophila*, the sufficient YA/YA genotype was significantly more frequent than in controls (OR 5.43; CI 1.32-22.41; p = 0.02). The frequency of the MBL deficient genotype was significantly higher in patients with viral (co-)infections (OR 2.36: CI 1.06-5.26; p = 0.03) and non-significantly higher in patients with pneumococcal pneumonia and viral (co-)infections. MBL genotypes had no effect on outcome.

Conclusions

MBL genotypes play a limited role in pneumococcal pneumonia. Sufficient MBL genotypes were more frequently found in a small group of patients with atypical pneumonia, and MBL deficient genotypes were more frequently found in patients with viral (co-)infections.

Introduction

Community-acquired pneumonia (CAP) is the most common infectious disease requiring hospitalization in the Western world. In spite of improving antibiotic regiments, CAP is still a disease with a significant mortality and morbidity [1]. CAP is caused by a variety of micro-organisms. Most frequently isolated micro-organisms are *Streptococcus pneumoniae*, *Haemophilus influenzae*, atypical pathogens (*Legionella pneumophila* and *Mycoplasma pneumoniae*) and influenza-viruses [2-6]. Although some micro-organisms are found in patients containing specific risk factors, such as Gram-negative stained bacteria in patients with a history of COPD, many patients suffer from CAP in absence of these risks. Genetic susceptibility to a specific pathogen causing CAP may play a role in these patients [7].

Mannose (or mannan) binding lectin (MBL) is a calcium dependent collagenous serum lectin produced by the liver during the acute phase response of inflammation. MBL binds to carbohydrates (mannose or N-acetylglucosamine) on the surface of different micro-organisms and is therefore called a pattern-recognition molecule. After binding, MBL mediates complement activation and opsonophagocytosis by activation of different mechanisms [8-14]. MBL deficiency is associated with an increased general risk of severe infection in immune compromised patients [15-19]. In other patient groups, MBL deficiency is associated with recurrent respiratory infections and infections with capsulated bacteria [20-22]. Furthermore MBL deficiency increases the risk of bacteraemia, sepsis and fatal outcome [24-27].

MBL deficiency is common and caused by single nucleotide polymorphisms (SNPs) in its promoter and coding regions on chromosome 10 (MBL) [28-32]. We investigated whether MBL polymorphisms are associated with the susceptibility to CAP in general and to CAP caused by the most frequent causative pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila* and *Mycoplasma pneumoniae*) and viral co-infections (Influenza A and B, and Herpes Simplex virus 1). Furthermore we report the effect of the MBL polymorphisms on clinical course.

Methods

Patients and controls

All patients (> 18 years) with CAP presenting in the period October 2004 - August 2006 in our general 600-bed teaching hospital were included in this study. Patients with a history of recent hospitalization (< 30 days) or a congenital or acquired immunodeficiency (including patients treated with prednisone 20 mg per day for more than three days) were excluded. Pneumonia was defined as a new infiltrate on the chest X-ray and two out of six clinical signs of pneumonia (cough, production of sputum, signs of consolidation on respiratory auscultation, temperature >38 or <35 degrees Celsius, leukocytosis (white blood count (WBC) >10 G/l) or leukopenia (WBC <4 G/l) or more than 10% rods in the differential count and C-reactive protein (CRP) 3 times above the upper limit of normal (5 mg/l). The chest X-ray was interpreted by the resident in the emergency department. Within 24 hour after presentation, the chest X-ray was evaluated by an experienced radiologist, who was not aware of the clinical course of the patient. The following data were collected at presentation: age, sex, history of COPD and Fine-score. A DNA-sample was taken at admission and stored for further analysis. The control group consisted of 223 Caucasian white sex-matched blood bank donors from the same geographical area as the patients. This study was approved by the local Medical Ethics Committee and informed consent was obtained from each patient.

Pathogen identification

Of all patients sputum (if available) and blood (two samples) were cultured. Polymerase chain reactions (Taqman real-time PCR) were performed in sputum in order to detect DNA of *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Chlamydophila pneumoniae* or *psittaci* [33]. Urine samples were taken for antigen testing on *Streptococcus pneumonia* (Binax *S. pneumoniae* kit) and *Legionella pneumophilia* (Binax Legionella urine antigen test) [34, 35]. Sampling for serologic testing on the presence of antibodies against *Mycoplasma pneumoniae*, *Coxiella burnetii* or respiratory viruses (influenza A and B and parainfluenza viruses 1, 2 and 3, adenovirus and respiratory syncytial virus) was done at day 1 and 10 (Complement fixation CBR), for *Legionella pneumophila* on day 1 and 30 (IgG/IgM virion/serion ELISA Clindia)[36]. Pharyngeal samples were taken for viral culture (influenza and parainfluenza viruses and herpes simplex virus type 1 (HSV1)). Viral pneumonia was defined in the presence of positive viral testing (culture or seroconversion) in combination with the presence of negative cultures, PCRs, serologic and antigen tests for bacterial micro-organisms.

Genotyping of MBL

Genotyping was done after closing of inclusions. Genotyping of *MBL2* (GeneID 4153) was done for the promotor X/Y SNP (rs7096206) and the exon 1 SNPs on codon 52 (rs5030737; D52C; D variant), 54 (rs5030737; G54D; B variant) and 57 (rs1800451; G57E; variant C). The promoter SNP is denoted as X/Y, with Y as wild type. The three coding SNPs in exon 1 D, B en C are all denoted by 0 versus wild type A. These SNPs are combined in 6 genotypes: YA/YA, YA/XA, XA/XA, YA/0, XA/0 and 0/0. MBL serum levels are sufficient in YA/YA, YA/XA, XA/XA and YA/O and deficient in XA/0 and 0/0. Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Kit (Qiagen, Westburg, the Netherlands). Combined MBL X/Y promoter and exon 1 genotypes were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay with slight modifications in a nested PCR protocol. Two PCR assays specific for the promotor X genotype (forward

primer ATT TGT TCT CAC TGC CAC C; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; 25 cycles, annealing temperature 63°C) or Y genotype (forward primer TTT GTT CTC ACT GCC ACG) were run. The PCR products were diluted 1:100 in distilled water. MBL exon 1 was amplified from these dilutions with an extra GC-clamp attached (forward primer with clamp CCG CCC GCC GCG CCC CGC GCC CGG CCC GCC GCC CCC GCC CCG TGT TCA TTA ACT GAG ATT AAC CTT C; reverse primer CAG AAC AGC CCA ACA CG). The amplified DNA was run overnight on a 6% polyacrylamide gel containing a denaturing gradient linearly increasing from 35% to 55% formamide and urea. All exon 1 genotypes had different patterns of migration. The corresponding X/Y promoter genotype could be inferred from the presence or absence of a product in the nested PCR.

Statistics

Statistical analysis for the frequencies of the different genotypes was done by Pearson's Chi-square tests or Fisher's exact tests. Logistic regression analysis was performed with univariate statistically significant results and age as a known risk for CAP. A difference of $p < 0.05$ was considered as being statistically significantly. All calculations were done by using SPSS version 11.0.

Results

Patients and controls

Initially 255 patients were considered eligible for this study; in 201 cases a new infiltrate on chest X-ray was confirmed by an experienced radiologist. In one patient DNA isolation failed (Gram-negative pathogen) and in one patient exon 1 analysis failed (unidentified micro-organism and XY promoter genotype), leaving 199 patients for further analysis. Patient characteristics are shown in Table 1. In 127 cases (64%) the causative micro-organism of CAP was identified. Most frequent identified micro-organisms were: *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Staphylococcus aureus*. Viruses were isolated in 33 patients, either as causative micro-organism of CAP (n = 16), or as co-infection (n= 17). The 223 controls were blood bank donors derived from the same geographical region as the patients.

MBL genotypes in patients and controls

Table 2 shows the frequency of the six MBL halpotypes and the two functional groups of genotypes, coding for sufficient (YA/YA, YA/XA, XA/XA and YA/0) and deficient (XA/0 and 0/0) MBL serum levels. As shown, frequencies did not differ between patients with CAP in general and controls. Furthermore, we found no differences in frequencies of MBL genotypes between sexes, ages (decades) and Fine-classes within patients, and between patients and controls.

Table 2 also shows the frequencies of MBL genotypes in patients with CAP caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila* and *Mycoplasma pneumoniae.* No differences were found in frequency of MBL genotypes between patients with CAP caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* and controls. The frequency of genotypes coding for sufficient MBL-levels was higher in patients with CAP caused by *Legionella pneumophila* and *Mycoplasma pneumonia* compared to controls, but this difference did not reach the level of statistical significance. The frequency of the YA/YA genotype in patients with Legionella pneumonia (n = 6/9, 67%) was significantly higher than in controls (n = 60/223, 27%): OR 5.43

(CI 1.32-22.41; p = 0.02). In the three patients in whom other atypical pathogens (*Chlamydophila* species and *Coxiella burnetii*) were isolated, one had a MBL deficient genotype. The six patients with pneumonia with *Staphylococcus aureus* consisted of 4 patients with a sufficient MBL genotype and 2 with a deficient genotype. The 72 patients with negative pathogen isolation consisted of 12 (17%) patients with a deficient and 60 (83%) with a sufficient genotype.

Viral pneumonia was diagnosed in 16 patients, but in 7 of these patients HSV1 was isolated and this is an uncommon causative micro-organism of CAP, leaving 9 patients for further analysis. Results of this analysis are also shown in Table 2. The frequency of MBL-deficiency was higher in patients with viral pneumonia (4/9; 44%) compared to controls (39/184; 18%), but this difference did not reach the level of statistical significance (p = 0.06). Pure influenza pneumonia was diagnosed in 6 patients, of which 2 were MBL-deficient (33%). Positive viral tests were present in 33 patients. Influenza (A and B) was the most frequently isolated and/or cultured virus and frequencies of the MBL genotypes in these patients are shown in the last column of Table 2. The frequency of deficient MBL genotypes was higher in patients with influenza, but this difference did not reach the level of statistical significance. In the 33 patients in which any virus was identified (influenza A and B, parainfluenza 1 and 3, HSV1, respiratory syncytial virus en adenovirus), the frequency of MBL deficient genotypes (n = 11/33, 33%) was significantly higher than in controls: OR 2.36 (CI 1.06-5.26); p = 0.03), but this difference did not remain statistically significant in logistic regression containing age and MBL genotype. In younger patients (< 55 year) with viral co-infections the frequency of MBL deficient genotypes was significantly higher compared to controls: 4/9 (44%) versus 20/138 (15%); OR 4.72 (CI 1.17-19.23; p = 0.04). MBL-deficiency was more often present in patients with the non-respiratory pathogen HSV1 (4/9; 44% versus 39/184; 18%; p = 0.06). In patients with CAP caused by *Streptococcus pneumoniae* and viral co-infection, the frequency of MBL-deficiency was 3/13 (23%) compared to 39/184 (18%) in controls and 7/40 (18%) in patients with pneumococcal pneumonia without viral co-infection, but these differences did not reach the level of statistical significance. Similar statistically non-significant results were found for patients with pneumococcal pneumonia and influenza A or B co-infection: MBL-deficiency was found 2/5 (40%) patients, 39/184 (18%) controls and 8/40 (17%) patients with pneumococcal CAP and negative influenza testing (non-influenza viruses or virus negative).

Table 1: Characteristics of 199 patients with CAP

Patient characteristics			
Male, n (%)		123	(62)
Age, mean (SD)		63	(17)
COPD, n (%)		61	(31)
Causative micro-organism, n (%)	Unidentified	72	(36)
	Streptococcus pneumoniae	60	(30)
	Haemophilus influenzae	14	(7)
	Legionella pneumophila	9	(5)
	Mycoplasma pneumoniae	9	(5)
	Staphylococcus aureus	6	(3)
	Other Gram-negative bacteria[1]	8	(4)
	Other atypical micro-organism[2]	3	(2)
	Other streptococcus	2	(1)
Positive viral testing, n (%)	Influenza-virus (A and B)	13	(7)
	Other respiratory viruses[3]	11	(6)
	Herpes simplex virus 1	9	(5)
Hospital mortality, n (%)		10	(5)
ICU admission, n (%)		21	(11)
Length of hospitalstay, median (range)		11	(3-153)
Bacteremia, n (%)		18	(9)
Fine-class, n (%)	I	30	(15)
	II	34	(17)
	III	53	(27)
	IV	55	(28)
	V	27	(14)

1 *Acinetobacter calcoaceticus, Escheridia Coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*

2 *Chlamydophila* species and *Coxiella burnetii*

3 adenovirus, para-influenzavirus 1 and 3, and respiratory syncytial virus

Table 2: Frequency of mannose-binding lectin genotypes in controls, patients with CAP, patients with CAP caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila*, *Mycoplasma pneumoniae* and viruses, and patients with CAP with positive viral testing for influenza-viruses (n, %).

Genotype	Controls (n = 223)	CAP (n = 199)	*S.pneumo.* (n = 60)	*H.influenzae* (n = 14)	*Legionella* (n = 9)	*Mycoplasma* (n = 9)	Viral (n = 9)	Influenza (n = 13)
YA/YA	60 (27)	55 (28)	16 (27)	3 (21)	6 (67)[3]	5 (56)	1 (11)	2 (15)
YA/XA	46 (21)	45 (23)	16 (27)	3 (31)	1 (11)	3 (33)	1 (11)	3 (23)
XA/XA	12 (5)	11 (6)	6 (10)	2 (14)	0	0	0	2 (15)
YA/0	66 (30)	51 (26)	12 (20)	4 (29)	1 (11)	1 (11)	3 (33)	2 (15)
XA/0	26 (12)	27 (14)	6 (10)	2 (14)	0	0	3 (33)	3 (23)
0/0	13 (6)	10 (5)	4 (7)	0	0	0	1 (11)	1 (8)
Sufficient[1]	184 (83)	162 (81)	50 (83)	12 (86)	9 (100)	9 (100)	5 (56)	9 (69)
Deficient[2]	39 (18)	37 (19)	10 (17)	2 (14)	0	0	4 (44)	4 (31)

1 A/A (YA/YA, YA/XA, XA/XA) + YA/0

2 XA/0 + 0/0

3 YA/YA versus non-YA/YA in patients with CAP caused by *Legionella pneumophila* compared with controls: OR 5.43 (CI 1.32-22.41; p = 0.02)

MBL genotypes and outcome

Mortality, number of patients admitted to the ICU or with positive cultures of blood (bacteraemia) did not differ between patients with a sufficient and deficient MBL genotype (Table 3). This also counts for length of hospital stay.

Table 3: Mortality, frequency of ICU admission and bacteraemia and length of hospital-stay in patients with CAP with sufficient and deficient mannose-binding lectin (MBL) genotypes (n, %)

MBL genotype	Mortality (n = 10)	ICU admission (n = 21)	Bacteraemia (n = 18)	Length of hospital-stay (median, range)
Sufficient (A/A+YA/0; n=162)	8 (5)	18 (11)	15 (9)	11 (3-69)
Deficient (XA/0+0/0; n=37)	2 (5)	3 (8)	3 (8)	11 (5-153)

Discussion

This study shows no role of MBL genotypes in pneumoccocal pneumonia, but a possible role in viral (co-) infections. MBL-sufficient genotypes were more often observed in a small group of patients with CAP caused by *Legionella pneumophila* and *Mycoplasma pneumoniae*. MBL genotypes had no effect on clinical outcome.

The role of MBL genotypes in invasive *Streptocoocus pneumonia* disease has been reported before [37, 38]. Laboratory findings suggest a minor role for MBL: MBL-binding to *Streptococcus pneumoniae* is weak [11]. The results of studies in patients with pneumococcal disease are conflicting; one study reports MBL deficiency as a risk for pneumococcal sepsis [38], which is not confirmed by a second study [37]. We did not find an increased risk for pneumococcal CAP in patients with deficient MBL genotypes. Genetic and geographical background of earlier two studies was similar to ours, but there are also many differences. The previously published studies have a retrospective design. Patients with positive blood cultures for *Streptcooccus pneumoniae* were genotyped, making them different from our patients by origin of the *Sterptococcus pneumoniae* (pneumococcal CAP versus pneumococcal bacteraemia) and the severity of disease (bacteraemia). Another explanation could be the presence of a (prior) viral (co-)infection. The frequency of MBL-deficiency in patients with positive viral testing was higher compared to controls and negative viral testing, especially for influenza, but these differences did not reach the level of statistical significance. The role of MBL genotypes in invasive *Streptococcus pneumoniae* infections remains unclear, but is limited in the susceptibility for community acquired pneumococcal pneumonia, and possibly only present in patients with a viral co-infection.

The number of patients with atypical pneumonia in our group is too small to reach levels of statistically significant differences. Earlier reports of *Mycoplasma pneumoniae* infections in children reported the contrary [39]. In *Legionella pneumophila* pneumonia MBL levels in serum tend to be low [40]. Although genotypes of these patients are unknown, it suggests the presence of deficient MBL genotypes. The complete absence of MBL-deficient genotypes in patients with *Legionella pneumophila* and *Mycoplasma pneumoniae* pneumonia in our study is remarkable. Possible explanations for the difference between this finding and ours are that low MBL serum levels are rather caused by utilization than by genotype or the presence of different serotypes of *Legionella pneumophila*. Another explanation could be that MBL deficiency is protective for atypical pneumonia due to the intracellular nature of bacteria causing atypical CAP. Both *Legionella pneumophila* and *Mycoplasma pneumoniae* share the ability to live intracellular, possibly making extra cellular binding of MBL impossible. The role of MBL deficiency in atypical pneumonia is a subject of further research.

The role of MBL genotypes in human viral infection in pneumonia is unknown. Interaction between viruses and MBL is best described for influenza A virus, which is inactivated by MBL, and there are similar reports for herpes simplex virus type 2 [41, 42]. We found tendencies for MBL-deficiency as a risk for viral pneumonia and co-infection, including pure influenza pneumonia and co-infection with influenza A and B. Similar results were found for patients in whom HSV1 was isolated. But HSV1 is not regarded as a causative micro-organism of CAP and positive testing was probably due

to re-activation. Positive viral testing in general showed a significant role for MBL genotypes. This significant difference disappeared in multivariate analysis, in which age was a far more predictor for viral co-infection compared to MBL insufficiency. Only in young patients a deficient MBL genotype is a risk for viral (co-)infection.

MBL genotypes had no effect on clinical course. This study was underpowered to show effect on the clinical endpoints death and admission to the ICU, but duration of stay in the hospital and positive blood cultures did not differ between the different genotypes. Main limitation of this study is sample-size. Although we succeeded to identify the causative micro-organism in a majority of the patients, the number of patients with atypical pneumonia and viral co-infections is too small and heterogeneous to find significant differences in frequency of genotypes. Similar counts for the number of patients with *Staphylococcus aureus*; in experimental setting MBL deficient mice are highly susceptible for *Staphylococcus aureus* pneumonia [43].
In summary, MBL genotypes possible play a pathogen dependent role in CAP. Contrary to previous reports, MBL genotypes play no role in pneumonia caused by *Streptococcus pneumoniae*. In a small group of patients with pneumonia with atypical, intracellular micro-organisms MBL-sufficient genotypes were more frequently found. Deficient genotypes were found more often in patients with viral (co-)infection, but not in multivariate analysis containing age. Only young patients with a deficient MBL genotype are at risk for viral co-infection. MBL genotypes have no effect on outcome.

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Summary and general discussion

Summary and general discussion

Introduction

This thesis is focused on the association between the variations in innate immunity and the susceptibility to and the clinical outcome of community-acquired pneumonia (CAP). In prior experimental studies, these associations have been limited to specific groups of micro-organisms. Part I of this thesis focuses on the identification of micro-organisms in patients with CAP. The prevalence of causative micro-organisms in a population of non-immunocompromised patients with CAP is reported, with an emphasis on the contributions of several diagnostic tools, easy accessible laboratory and clinical parameters and pathogen-specific risk factors for CAP. Based on these results, a diagnostic protocol for pathogen identification in CAP is proposed.

In the Intermezzo (the transition between Part I and Part II), the association between ACE-polymorphisms and the susceptibility to as well as the clinical outcome of CAP is described. The relationship between ACE-polymorphisms and the decline in ACE serum levels during CAP is also studied.

Part II deals with the role of genetic variations within three different components of the innate immune system: cytokines, receptors and the complement system.

Part I: Clinical characteristics

In Chapter 1, the causative micro-organisms in 201 adult non-immunocompromised patients with CAP are described. In 64% of the patients, a pathogen was found. Consistent with previous reports, *Streptococcus pneumoniae* was the most frequently identified causative micro-organism (49%), followed by *Haemophilus influenzae* (11%), *Legionella pneumophila* (7%) and *Mycoplasma pneumoniae* (7%). Several diagnostic tools were evaluated, after which a three-step diagnostic protocol was constructed. The first step of this protocol consists of cultures of sputum and blood in combination with Legionella and pneumococcal urine antigen tests. In the event of negative results, the second step consists of polymerase chain reaction (PCR) of sputum samples to detect *Chlamydophila pneumoniae* and *psittaci*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*. By using this protocol, all clinical relevant causative micro-organisms are identified by the first two steps within 48h after admission. Most international guidelines for the treatment of CAP recommend pathogen-directed antibiotic therapy. The increasing resistance of respiratory bacteria for many different antibiotics, especially outside the Netherlands and Scandinavia, is also a strong argument in favour of pathogen identification. The proposed diagnostic protocol is currently evaluated in a prospective cohort study.

Failure of pathogen identification occurs in 36% of the patients, and the clinical characteristics of these patients are presented in Chapter 2. As expected, the use of antimicrobial therapies at admis-

sion is associated with a failure of pathogen-identification. A relatively low C-reactive protein (CRP) at admission is also an independent predictor of failure of pathogen-identification, possibly due to the presence of viral or atypical pathogens with low inflammatory properties. The same is true for a history of hypertension, though the meaning of this finding remains unclear.

Chapter 3 describes the influence of prior antibiotic treatment on the yield of identified micro-organisms that can be identified. In patients previously treated with beta-lactam antibiotics, the frequency of atypical pneumonia is higher when compared to patients without outpatient therapy with beta-lactam antibiotics. The use of macrolides during the weeks before admission predicts viral pneumonia, though one may doubt whether these patients truly suffer from viral pneumonia only or from mixed bacterial-viral pneumonia that gives negative diagnostic results with different methods of bacterial identification (culture, urine antigen testing and PCR). Mixed bacterial-viral infections are observed in 16% of the patients, especially influenza in combination with pneumococcal pneumonia. The role of these respiratory viruses in the pathogenesis of (pneumococcal) CAP is unknown. It is possible that the viral infection is the primary infection damaging the muco-ciliary barrier of the upper airways. Alternatively, it is a co-infection that up regulates the inflammatory response. The possible presence of a large number of viruses in the sputum of this population is currently being analysed.
Chapter 4 describes the clinical characteristics of patients with the two most frequently identified causative micro-organisms of CAP, *Streptococcus pneumoniae* and *Haemophilus influenzae*, as well the clinical characteristics of patients with atypical CAP caused by the *Chlamydophila* species, *Coxiella burnetii*, *Legionella pneumophila* and *Mycoplasma pneumoniae*. Patients with atypical pneumonia are characterised by high fever, hyponatraemia and hypokalaemia. Hypokalaemia is probably due to respiratory alkalosis, a frequently observed phenomenon in patients with *Legionella pneumophila* pneumonia. Different clinical characteristics are found between patients with pneumococcal and non-pneumococcal pneumonia, and between patients with atypical and non-atypical pneumonia. However, the sensitivity and specificity of the clinical characteristics of pneumococcal and atypical pneumonia are low. They can serve only as additional variables in the choice for initial antibiotic therapy. In the diagnostic protocol proposed in Chapter 1, these patients would have been identified within 48h after admission.

Chapter 4 further identifies other risk factors for CAP in specific groups of bacteria. Patients with CAP caused by *Haemophilus influenzae* are old (median 75 years) and have a medical history of COPD. On the other hand, patients with CAP caused by atypical bacteria are young and lack a history of COPD in their medical history.

Intermezzo: ACE polymorphisms

The use of ACE (angiotensin converting enzyme)-inhibitors results in low levels of circulating ACE and has also been reported to decrease the susceptibility to CAP in Asian and white diabetic patient populations. The reasons for this protective effect of ACE-inhibitors are thought to be due to an improved removal of secretion from the upper airways, a better cough reflex, and immunomodulation by a decrease in angiotensin II levels. It is also possible that the use of ACE-inhibitors reflects a generally higher standard of care for populations at risk for CAP (diabetics, patients with congestive

heart failure). In Asians, the ACE D allele (high levels of serum ACE) is associated with increased susceptibility to CAP. However, no relationship between ACE polymorphisms and susceptibility to CAP is found in our population of Dutch Caucasians. It seems that this association is limited to Asians only (Chapter 5).

Low ACE levels have been reported previously in the course of adult respiratory distress syndrome (ARDS) and pneumonia, but these levels have not been associated with ACE I/D polymorphism. The ACE I/D polymorphism regulates ACE serum levels: the highest ACE levels are found in the ACE DD genotype, and the lowest levels are found in the ACE II genotype (Chapter 6). As previously reported in ARDS and pneumonia, ACE levels in CAP are low during hospitalisation and show a further decline during the first days of a hospital stay. ACE levels can be correlated with disease severity, which is reflected by the highest APS scores. Although the clinical relevance of these findings is limited, these observations are nonetheless interesting. A possible explanation is the destruction of the pulmonary vasculature, the main source of ACE, but most of the patients in our population only suffered from lobular pneumonia with restricted loss of pulmonary vasculature. Increased demand for angiotensin II may be another explanation; an increase in angiotensin II has been observed in sepsis, and this is in agreement with the previous finding that patients with more severe disease had lower ACE serum levels. In these patients, the renin-angiotensin system (RAAS) would be up-regulated due to intravascular volume depletion, which results in tachycardia, hypotension and oliguria. To restore circulating volume, RAAS may be activated to increase the conversion of angiotensin II from angiotensin I, and this may further result in a depletion of ACE stores. Confirmation of this theory may be found in a (non-pulmonary) population of patients with sepsis.

Part 2: Innate immunity

Numerous genetic variations within the three examined components of the innate immune system are found in patients with CAP. These variations are associated with the degree of susceptibility to CAP in general, the specific patient group or the specific pathogen. Genetic variations are sometimes associated with the clinical outcomes of CAP.

In Chapters 7 and 8, the association between the functional polymorphisms in interleukin-6 (Chapter 7) and interleukin-10 (Chapter 8) as well as the susceptibility to CAP are reported. The interleukin-6 (IL-6) -174 CC genotype is associated with an increased susceptibility to severe CAP (Chapter 7). This genotype results in a decreased inflammatory response. A possible explanation may be that patients with the IL-6 -174 CC genotype have a limited initial inflammatory response. This may result in a higher bacterial load, which further results in more severe CAP. In patients presenting with severe CAP, the frequency of positive blood cultures is higher when compared to either patients with non-severe CAP or to IL-$6^{-/-}$ mice. The bacterial load is also higher when compared to the control mice. The association between the IL-10 AAG haplotype, which is more frequent in young patients with CAP, and IL-10 levels is not yet fully understood. However, recent reports have suggested that this haplotype is associated with an increased IL-10 response after stimulation with pneumococcus (Chapter 8). This is in line with our findings in IL-6, as IL-10 is a cytokine with mainly anti-inflammatory properties. Up-regulation of IL-10, as suggested in the IL-10 AAG haplotype, may result in a

decrease in the initial inflammatory response and further result in CAP requiring hospitalisation. The IL-10 AAG haplotype is only associated with an increased susceptibility to CAP in young patients. In the elderly, other risk factors for CAP, including age, congestive heart failure and COPD may minimise the effect of genetic polymorphisms. To increase the power of the analysis, new samplings to determine IL-6 and IL-10 polymorphisms are currently performed in the Ovidius trial.

IL-6 and IL-10 polymorphisms are not associated with the clinical outcome of CAP, regardless of IL-6 and IL-10 serum levels. In contrast to CRP and WBC (Chapters 7 and 8), IL-6 and IL-10 are both acute phase proteins and are related to the clinical course. An excess of inflammation is associated with severe clinical course. A simple explanation may be that this is only a reflection of the severity of disease. However, high levels of both IL-6 and IL-10 *at admission* are already associated with future complications during hospitalisation. It is the excessive response itself that may be responsible for a more detrimental clinical course, and this phenomenon has been reported previously in ICU patients. There are also some preliminary studies showing that a decrease in the inflammatory response by the use of corticosteroids improved the clinical outcome of patients with pneumonia. This intervention has been shown to be successful in patients with sepsis, meningitis and tuberculosis. This is an interesting topic and forms the basis for an ongoing, prospective study (the Ovidius trial) in our region. The Ovidius trial is a blind-placebo controlled intervention study about the effect of dexamethason in CAP.

In Chapter 9, the associations among the functional polymorphisms of the genes coding for Toll-like receptor (TLR) 2, TLR4 and TLR5 are reported. Contrary to cytokine polymorphisms, the association between TLR polymorphisms and CAP is limited to a specific pathogen. This is due to the nature of TLR, which all have the binding capacity for a limited group of micro-organisms. The only exception is the TLR2 753 A allele (decreased binding capacity of TLR2), which is not only associated with an increased susceptibility to CAP in general but even more so to CAP caused by *Haemophilus influenzae* and other Gram-negative bacteria. TLR2 is reported to have binding capacity for the cell wall components of Gram-negative bacteria. The TLR5 392stop genotype is associated with an increased susceptibility to CAP caused by *Streptococcus pneumoniae*. This is an interesting observation. So far, only TLR2 and TLR4 are recognised as receptors for *Streptococcus pneumoniae*. However, the absence of TLR2 or TLR4 is not associated with detrimental effects in an experimental setting. TLR5 is capable of binding flagellin, a part of *Legionella pneumophila*. It may be possible that TLR5 also recognises a part of the pneumococcus, which is a subject of further experimental research. As reported previously, TLR4 and TLR5 are involved in the susceptibility to Legionella pneumonia.

The Fcγ-RIIa R131 genotype, which codes for decreased binding of IgG2, is associated with CAP caused by *Haemophilus influenzae* (Chapter 10). Next to TLR2 A allele (Chapter 9), this is the second polymorphism associated with CAP caused by *Haemophilus influenzae*. As reported in Chapter 4, COPD is a risk factor for *Haemophilus influenzae* pneumonia. Though the number of patients in this population with CAP caused by *Haemophilus influenzae* is too small to draw definite conclusions, it may be interesting to identify the frequency of these polymorphisms in patients with COPD and to explore its association with *Haemophilus influenzae* infections in this group of patients. In Chapter 10, it is further reported that the Fcγ-RIIa R131 genotype is also associated with severe

sepsis, most often due to pneumococcal pneumonia. The involvement of the Fcγ-RIIa receptor in invasive pneumococcal disease has been reported before. Together with the TLR5 392stop genotype, the Fcγ-RIIa R131 genotype is associated with invasive (pneumococcal) disease in CAP. Currently, this association is further explored in the Ovidius trial. These two polymorphisms may predict the susceptibility for acquiring invasive pneumococcal disease, and it may be interesting to screen populations at risk for the presence of these polymorphisms.

Chapter 10 reports a significantly *lower* frequency of the Fcγ-RIIa R131 genotype in patients with pneumonia caused by *Mycoplasma pneumoniae*. Similar observations are found for mannose-binding lectin (MBL) genotypes that code for normal MBL serum levels (Chapter 12). These genotypes are also associated with an increased susceptibility to CAP caused by *Mycoplasma pneumoniae*. The same has been found for another intracellular living bacterium, *Legionella pneumophila*. It seems that both types of facultative intracellular living bacteria benefit from the presence of normal genotypes of Fcγ-RIIa receptor and MBL. The presence of these normal genotypes potentially facilitates opsonophagocytosis, thereby allowing these organisms to temporarily escape from detection or signalling by the immune system. This is also why Legionella pneumophila was recently called “Mr. Hyde” in a review on the pathogenesis of Legionnaires diseases. This may also hold true for other intracellular living micro-organisms, including parasites. Furthermore, this may also be a possible explanation for why there is a high frequency of MBL deficiency, and therefore protection for these micro-organisms, in Africa. This is a subject of ongoing research.

In Chapter 11, the relationship between MBL genotypes and MBL serum levels is reported. MBL acts as an acute phase protein, and MBL serum levels are dependent on the MBL genotype. Serum levels are very low to absent in the XA/XA, XA/O and O/O genotypes, and these MBL genotypes are regarded as being MBL deficient. In patients with MBL-sufficient genotypes, their MBL serum levels vary widely. However unlike patients with MBL-deficient genotypes, those with sufficient MBL genotypes usually have their MBL levels return to normal after recovery from pneumonia. In the past, MBL deficiency has been defined by the MBL serum level in the acute phase, which is only partly confirmed by our findings (e.g., i.e. only in MBL deficient genotypes). Whether absent or very low MBL serum levels in MBL-sufficient genotypes are a result of consumption or an inability of production is a subject of ongoing research, as is the relevance of MBL genotypes and serum levels with regard to the phagocytotic function of MBL.

MBL-deficient genotypes are associated with an increased susceptibility for acquiring viral infection in CAP (including viral co-infection in pneumococcal pneumonia), but not for pneumococcal CAP itself, as was previously reported (Chapter 12). A possible explanation for this difference in findings may be a difference in the study design between that of previous studies and ours. Previous studies on the association between MBL polymorphisms (and other polymorphisms in innate immunity) performed a retrospective design: patients were included on the basis of positive blood cultures during hospitalisation. On the other hand, this thesis contains the results of a prospective cohort of patients with CAP, including patients with positive blood cultures. The results of this study pertain to patient susceptibility for acquiring (pathogen-specific) CAP, whereas previous reports pertain to the susceptibility for acquiring invasive diseases (defined as positive cultures of blood) by hospitalised patients. This also explains the differences between the results presented in Chapter 12 and that of

previous reports on the association between MBL deficiency and invasive *Streptococcus pneumoniae* disease. Furthermore, *Streptococcus pneumoniae* only binds weakly to MBL, but it binds strongly to influenza virus. This may explain our findings in viral pneumonia and viral co-infection. MBL also binds to *Staphylococcus aureus*, an observation reported in Chapter 12. As *Staphylococcus aureus* is an infrequent causative agent of CAP, we are currently testing the relationship between MBL deficiency and staphylococcal infections in a population of patients with staphylococcal peritonitis who are on continuous ambulatory peritoneal dialysis (the Thales project).

General consideration

Streptococcus pneumoniae and *Haemophilus influenzae* are the most frequently identified micro-organisms in CAP. Patient characteristics, including clinical characteristics and history of prior antibiotic use, contribute to the failure of identifying the pathogens and the causative micro-organisms in CAP. A three-step diagnostic protocol is presented, in which by the time the first two steps are completed all clinical relevant micro-organisms are identified within 48h after patient admission through sputum and blood cultures, urine antigen test and PCR of the sputum for *Chlamydophila* species, *Legionella pneumophila* and *Mycoplasma pneumoniae*. The role of the ACE I/D polymorphism is limited in CAP. Low levels of ACE in serum are associated with the disease severity, which is reflected by the clinical scores. Within innate immunity, there are many genetic variations that are associated with the susceptibility to and outcome from pathogen-specific CAP. IL-6 and IL-10 polymorphisms are associated with an increased susceptibility of acquiring severe CAP or CAP in young patients. IL-6 and IL-10 are markers as well as predictors of disease severity. The TLR2 A allele and the Fcγ-RIIa R131 genotype are associated with an increased susceptibility to *Haemophilus influenzae* CAP, while TLR5 392stop and Fcγ-RIIa R131 are associated with an increased susceptibility to (severe) pneumococcal pneumonia. Fcγ-RIIa H131 (wild type) and MBL sufficient genotypes are associated with an increased susceptibility to CAP caused by *Legionella pneumophila* or *Mycoplasma pneumoniae*. MBL genotypes code for MBL serum levels, and MBL deficiency is associated with an increased susceptibility for acquiring additional viral infections in patients with existing CAP. Thus, the answer to the central question of this thesis - are variations in innate immunity are associated with the susceptibility to and outcome of CAP - is yes, but only in a pathogen-specific manner.

Samenvatting in het Nederlands

Samenvatting in het Nederlands

Inleiding

Dit proefschrift beschrijft de relatie tussen CAP (community-acquired pneumonia of buiten het ziekenhuis opgelopen longontsteking) en het humorale afweersysteem, waarbij de nadruk ligt op de gevoeligheid voor en klinisch beloop van de pneumonie. Uit eerdere, experimentele studies is bekend dat deze relatie zich beperkt tot specifieke micro-organismen. In Deel I van dit proefschrift wordt de identificatie van deze micro-organismen beschreven. Tevens wordt het vóórkomen van de verschillende micro-organismen bij patiënten met een normaal afweersysteem beschreven. Verder wordt er aandacht besteed aan de diverse diagnostische technieken, de rol van eenvoudige laboratoriumbepalingen, specifieke klinische kenmerken en micro-organisme specifieke risicofactoren voor een pneumonie. Op basis van deze resultaten wordt een voorstel gedaan voor een diagnostisch protocol bij een pneumonie.

In het intermezzo (de overgang tussen Deel I en Deel II) wordt de relatie beschreven tussen polymorfismen (variaties in de genen) in het ACE-gen en de vatbaarheid voor en het klinisch beloop van een pneumonie. De relatie tussen ACE polymorfismen en de kinetiek van ACE serum spiegels tijdens een pneumonie wordt tevens onderzocht.

Deel II van dit proefschrift behandelt de rol van genetische variaties in drie verschillende onderdelen van het humorale afweersysteem: cytokines, receptoren en het complement systeem.

Deel I: Klinische kenmerken

In Hoofdstuk 1 worden de verwekkers van een pneumonie beschreven in 201 patiënten met een normaal afweersysteem. Bij 64% van deze patiënten kan een verwekker worden aangetoond. De meest frequente verwekker is *Streptococcus pneumoniae* (bij 49% van de patiënten), gevolgd door *Haemophilus influenzae* (11%), *Legionella pneumophila* (7%) en *Mycoplasma pneumoniae* (7%). Na evaluatie van verschillende diagnostische technieken wordt een driestaps diagnostisch protocol geconstrueerd. De eerste stap in dit protocol bestaat uit kweken van sputum en bloed in combinatie met urine antigen testen voor Legionella en pneumokokken. Indien hierbij geen verwekker wordt aangetoond, volgt stap 2, die bestaat uit een PCR (polymerase chain reaction) van het sputum op *Chlamydophila pneumoniae* en *psittaci*, *Legionella pneumophila* en *Mycoplasma pneumoniae*. Door gebruik te maken van dit protocol kunnen op basis van onze theorie alle klinisch relevante verwekkers van een pneumonie binnen 48 uur na opname aangetoond worden. Daarmee sluit dit model goed aan op de meeste internationale richtlijnen voor de behandeling van een pneumonie, waarbij geadviseerd wordt om de behandeling met antibiotica zo snel mogelijk te richten op de verwekker van de pneumonie. De toename van antibioticaresistentie, met name buiten Nederland en Scandinavië, is een belangrijke reden om de verwekker van een pneumonie te identificeren. Daarmee kan

onnodig gebruik van breed-spectrum antibiotica voorkomen worden. Het beschreven diagnostische protocol wordt momenteel geëvalueerd in een prospectieve cohort studie.

Bij 36% van de patiënten met een pneumonie blijkt identificatie van de verwekker niet mogelijk te zijn. De klinische karakteristieken van deze patiënten staan vermeld in Hoofdstuk 2. Het is niet geheel onverwacht, dat het gebruik van antibiotica voorafgaand aan de ziekenhuisopname geassocieerd is met het uitblijven van identificatie van de verwekker. Ook een laag CRP (C-reactive protein) is een onafhankelijke voorspeller voor het falen van identificatie van de verwekker van een pneumonie. Dit is mogelijk het gevolg van de aanwezigheid van weinig pathogene, maar moeilijk te identificeren virussen en atypische micro-organismen. Een voorgeschiedenis van hypertensie is ook een onafhankelijke voorspeller van falen van identificatie van de verwekker van pneumonie. Wij hebben geen goede verklaring voor dit laatste fenomeen.

In Hoofdstuk 3 wordt het effect beschreven van antibioticagebruik voorafgaand aan de ziekenhuisopname op het type micro-organisme geïdentificeerd als de verwekker van de pneumonie. Bij patienten die voorafgaand aan hun ziekenhuisopname worden behandeld met beta-lactam antibiotica worden vaker atypische micro-organismen gevonden. Bij patiënten, thuis behandeld met macrolides, worden vaker virussen gevonden als verwekker van de pneumonie. Het is echter de vraag of deze laatste categorie patiënten daadwerkelijk alleen een virale pneumonie hebben; het is ook mogelijk dat er sprake is van een gecombineerde virale en bacteriële infectie met negatieve testresultaten voor bacteriën (kweken, urine antigen testen en PCR). Gecombineerde virale en bacteriële infecties worden gevonden bij 16% van de patiënten met een pneumonie; de meest voorkomende combinatie is het influenza virus en de pneumokok. De rol van respiratoire virussen in de pathogenese van (pneumokokken) pneumonie is niet bekend. Het is mogelijk dat initieel sprake is van een virale infectie, waarbij beschadiging van de barrière van mucosa- en trilhaarepitheel ontstaat en een bacteriële superinfectie optreedt. Verder is het mogelijk dat de virale infectie de inflammatoire respons versterkt met als gevolg een ernstiger klinisch beloop, waardoor patiënten zich eerder presenteren bij de huisarts. Momenteel wordt de aanwezigheid van virussen in het sputum van patiënten met een luchtweginfectie verder geanalyseerd.

Hoofdstuk 4 beschrijft de klinische karakteristieken van de patiënten met een pneumonie met de twee meest voorkomende verwekkers – *Streptococcus pneumoniae* en *Haemophilus influenzae* – en de klinische karakteristieken van patiënten met een atypische pneumonie veroorzaakt door *Chlamydophila species*, *Coxiella burnetii*, *Legionella pneumophila* of *Mycoplasma pneumoniae*. De belangrijkste klinische karakteristieken van deze laatste groep patiënten zijn hoge koorts, hyponatriëmie en hypokaliëmie. De hypokaliëmie is waarschijnlijk het gevolg van de respiratoire alkalosis, een frequente bevinding in patiënten met een *Legionella pneumophila* pneumonie. Hoewel de klinische karakteristieken verschillen tussen patiënten met en zonder pneumokokken pneumonie, en tussen patiënten met en zonder atypische pneumonie, zijn deze verschillen te klein om te gebruiken als sensitieve en specifieke voorspellers voor de verwekker van de pneumonie. Wel kunnen ze gebruikt worden als extra informatie bij het maken van de keuze voor initiële antibiotische therapie. Gebruikmakend van het protocol zoals beschreven in Hoofdstuk 1 zullen pneumokokken, *Haemophilus influenzae* of atypische bacteriën binnen 48 uur na opname geïdentificeerd zijn.
Hoofdstuk 4 beschrijft ook de risicofactoren voor een pneumonie veroorzaakt door een specifieke

verwekker. Hoge leeftijd en een voorgeschiedenis van COPD zijn risicofactoren voor een pneumonie veroorzaakt door *Haemophilus influenzae*. Hier tegenover staat dat jonge patiënten zonder COPD een hoger risico hebben op een pneumonie veroorzaakt door atypische bacteriën.

Intermezzo: ACE polymorphismes

Het gebruik van ACE (angiotensin converting enzyme) remmers resulteert in lagere spiegels van circulerend ACE. ACE remmers zijn geassocieerd met een lager risico op een pneumonie bij Aziaten en blanke patiënten met diabetes mellitus. Dit beschermende effect wordt toegeschreven aan een verbetering van de evacuatie van slijmvliessecreet, een verbeterde hoestreflex en modulatie van het afweersysteem. Bij Aziaten is het ACE D allel, dat codeert voor hoge spiegels van circulerend ACE, geassocieerd met een hoger risico op het krijgen van een pneumonie. In onze Nederlandse (Kaukasische) populatie zijn deze relaties niet aanwezig. De relatie ACE polymorphisme en vatbaarheid voor pneumonie lijkt zich dan ook te beperken tot Aziatische patiënten of populaties 'at risk' (Hoofdstuk 5).

Lage spiegels van circulerend ACE zijn kortgeleden beschreven bij patiënten met ARDS (adult respiratory distress syndrome) en pneumonie, maar deze spiegels zijn niet gecorrigeerd voor het onderliggende ACE polymorphisme. Het ACE I/D polymorphisme regelt namelijk de hoogte van de serum ACE spiegel: hoge spiegels in het DD genotype en lage spiegels in het II genotype (Hoofdstuk 6). Bij patiënten met ARDS of pneumonie in onze studiegroep zijn ACE spiegels laag bij opname met nog een verdere daling tijdens de eerste dagen van opname. De hoogte van de ACE serum spiegels is gecorreleerd aan de ernst van ziekte uitgedrukt in de APS (Acute Physiology Score). Hoewel de klinische impact van deze resultaten vooralsnog niet groot is, zijn ze wel interessant. Een mogelijke verklaring voor de lage ACE spiegels bij opname en bij ernstig zieke patiënten, is destructie van de pulmonale vasculatuur (waar ACE voornamelijk gemaakt wordt). Echter, de infiltratieve afwijkingen in de longen van onze patiënten beperken zich meestal tot eenzijdig en lobair. Een andere verklaring voor de lage ACE serum spiegels bij ernstig zieke patiënten zou een verhoogd verbruik van ACE ten gevolge van een verhoogde behoefte aan angiotensinogeen II kunnen zijn. Dit is eerder aangetroffen bij patiënten met sepsis. Dit is in overeenstemming met de eerder gevonden lage ACE serum spiegels bij ernstig zieke patiënten. In deze patiëntengroep is het RAAS (renin-angiotensinogen system) geactiveerd ten gevolge van intravasale volume depletie. Binnen het RAAS wordt angiotensinogeen I omgezet naar angiotensinogeen II door ACE. Een verhoogde activiteit van dit RAAS zou door verbruik kunnen resulteren in lagere ACE serum spiegels. Deze theorie zal echter bevestigd moeten worden in een populatie patiënten met sepsis (van extrapulmonale origine).

Deel II: Humorale afweersysteem

Bij patiënten met een pneumonie zijn diverse genetische variaties binnen de onderzochte delen van het humorale afweersysteem gevonden. Deze variaties zijn geassocieerd met vatbaarheid voor een pneumonie in het algemeen, in een specifieke groep patiënten of een specifieke groep verwekkers. Sommige genetische variaties zijn gerelateerd aan het beloop van een pneumonie.

In Hoofdstuk 7 en 8 wordt de relatie tussen functionele polymorfismen in de genen van interleukine 6 (Hoofdstuk 7), interleukine 10 (Hoofdstuk 8) en de vatbaarheid voor pneumonie beschreven. Het interleukine 6 (IL-6) -174 CC genotype is geassocieerd met een hoger risico op het krijgen van een ernstige pneumonie. In de literatuur is dit genotype geassocieerd met een verminderde inflammatoire respons. Een mogelijke verklaring voor het verhoogde risico op een ernstige pneumonie in patiënten met het -174 CC genotype is dat de initiële inflammatoire respons verlaagd is, hetgeen resulteert in een hoger aantal patiënten met bacteriemie en bijpassende klinische verschijnselen. Inderdaad is de frequentie van bacteriemie in patiënten met een ernstige pneumonie en in patiënten met het IL-6 -174 CC genotype verhoogd. In een experimentele setting is het klinisch beloop van IL-$6^{-/-}$ ('knock-out') muizen ook ernstiger dan bij controle muizen. Het IL-10 AAG haplotype is geassocieerd met een hogere vatbaarheid voor pneumonie bij jonge patiënten. Dat dit alleen geldt voor jongere patiënten kan mogelijk verklaard worden door het feit dat oudere patiënten andere risicofactoren (COPD, hartfalen, hoge leeftijd) hebben voor het ontwikkelen van een pneumonie, waardoor het effect van dit haplotype overschaduwd kan worden. Het IL-10 AAG haplotype is geassocieerd met een verhoogde IL-10 respons. Omdat IL-10 een interleukine is met vooral anti-inflammatoire eigenschappen, zou de gebrekkige initiële pro-inflammatoire respons van de patiënt na infectie kunnen resulteren in een meer ernstige pneumonie, zoals bij patiënten met het IL-6 -174 CC genotype. Momenteel wordt in een prospectieve studie (Ovidius studie) verder genetisch materiaal verzameld om de relaties tussen IL-6 en IL-10 polymorfismen en de vatbaarheid voor pneumonie verder te onderzoeken.

In tegenstelling tot IL-6 en IL-10 polymorphismes zijn IL-6 en IL-10 serum spiegels wel geassocieerd met het klinisch beloop van een pneumonie. IL-6 en IL-10 beide acute fase proteïnen die gerelateerd zijn aan het klinisch beloop (Hoofdstuk 7 en 8). Een teveel aan inflammatie is gerelateerd aan een ernstig klinisch beloop. Een eenvoudige verklaring zou zijn dat dit louter een reflectie is van de ernst van ziekte. Echter, de IL-6 en IL-10 serum spiegels zijn direct bij opname al gerelateerd aan complicaties in het verdere beloop van de opname. Deze observatie suggereert dat de excessieve inflammatoire respons zelf verantwoordelijk of voorspellend is voor een gecompliceerd en ernstig klinisch beloop. Dit is reeds eerder gevonden bij intensive care patiënten. Uit eerdere studies blijkt dat het onderdrukken van de inflammatoire respons door corticosteroïden het klinisch beloop gunstig beïnvloedt in patiënten met een pneumonie. Deze behandeling is eerder ook met succes toegepast bij patiënten met sepsis, meningitis of tuberculosis. Dit interessante onderwerp vormt de basis van een thans lopende prospectieve studie, waarbij dexamethason wordt vergeleken met placebo in een populatie patiënten met een pneumonie (Ovidius studie).
In Hoofdstuk 9 worden de relaties tussen functionele polymorfismen in de genen van de Toll-like receptoren (TLR) 2, TLR4 en TLR5 en pneumonie beschreven. In tegenstelling tot polymorfismen in de genen van cytokines zijn de relaties tussen TLR polymorfismen en het ontstaan van een pneumonie beperkt tot pneumonie veroorzaakt door een specifieke bacterie. Dit is het gevolg van de bindingscapaciteit van de TLRs; deze is allen van toepassing bij een beperkt aantal micro-organismen. De enige uitzondering is aanwezigheid van het TLR2 2477 A allel, coderend voor verminderde bindingscapaciteit van TLR2, dat niet alleen geassocieerd is met een groter risico op een pneumonie veroorzaakt door *Haemophilus influenzae* en andere Gram-negatieve bacteriën, maar ook met een groter risico op een pneumonie in het algemeen. Het TLR5 392stop genotype is gerelateerd aan een groter risico op een pneumonie veroorzaakt door *Streptococcus pneumoniae*. Dit is een interes-

sante bevinding, omdat tot nu toe alleen TLR2 en TLR4 bindingscapaciteit aan de *Streptococcus pneumoniae* toegedicht werd. In een experimentele setting blijkt echter dat afwezigheid van deze twee TLRs niet leidt tot een ernstig klinisch beloop bij pneumokokken ziekte. TLR5, tot nu toe met name geïdentificeerd als een flagelline binder, kan mogelijk ook vooralsnog onbekende structuren van de pneumokok binden. Dit is een onderwerp voor verder onderzoek. Naast deze nieuwe bevindingen, wordt de relatie tussen TLR4 en TLR5 polymorfismen en Legionella pneumonie bevestigd.
Het Fcγ-Receptor(R)IIa R131 genotype, coderend voor verminderde IgG2 binding, is geassocieerd met een verhoogde kans op een pneumonie veroorzaakt door *Haemophilus influenzae* (Hoofdstuk 10). Naast het TLR2 A allel (Hoofdstuk 9), is dit het tweede polymorfisme dat geassocieerd is met pneumonie veroorzaakt door *Haemophilus influenzae*. Zoals gemeld in Hoofdstuk 4, is COPD een risico voor het ontwikkelen van een pneumonie met *Haemophilus influenzae*. Het aantal patiënten met pneumonie veroorzaakt door *Haemophilus influenzae* in deze studie is te klein om conclusies te trekken, maar het zou interessant kunnen zijn om de frequentie van deze twee polymorfismen te onderzoeken in patiënten met COPD, waarbij vooral mogelijke relaties met exacerbaties ten gevolge van *Haemophilus influenzae* infecties vooral het onderzoeken waard zijn. In Hoofdstuk 10 wordt verder gemeld dat het Fcγ-RIIa R131 genotype geassocieerd is met ernstige sepsis, met name in het geval van pneumokokken pneumonie. Deze relatie is eerder gevonden. Samen met het TLR5 392stop genotype is het Fcγ-RIIa R131 genotype geassocieerd met ernstige pneumokokken pneumonie. Ook deze associatie onderzocht worden in de Ovidius studie. Deze twee polymorfismen zouden patiënten met een verhoogd risico op ernstige pneumokokken ziekte kunnen identificeren; bij bevestiging van deze associaties in een grotere onafhankelijke patiënten cohorten zou screening van kwetsbare patiënten populaties een mogelijkheid kunnen zijn.

Hoofdstuk 10 meldt verder een lagere frequentie van het Fcγ-RIIa R131 genotype in patiënten met pneumonie veroorzaakt door *Mycoplasma pneumoniae*. Soortgelijke observaties worden gedaan in patiënten met MBL (mannose-binding lectin) genotypen die coderen voor normale MBL serum spiegels (Hoofdstuk 12). Ook deze genotypen zijn geassocieerd met een hoger risico op een pneumonie veroorzaakt door *Mycoplasma pneumoniae* en voor een ander intracellulair levend micro-organisme, *Legionella pneumophila*. Het lijkt erop dat beide intracellulair levende bacteriën gebaat zijn bij de aanwezigheid van normale Fcγ-RIIa receptor en MBL genotypen. Deze genotypen faciliteren opsonophagocytosis, waardoor het voor deze bacteriën gemakkelijker wordt om zich in de cel te verborgen te houden en daarbij tijdelijk te ontsnappen aan het afweersysteem. Dit is de reden dat *Legionella pneumophila* in een recent overzichtsartikel over de pathogenese van Legionella infecties wordt aangeduid als “Mr. Hyde”. Mogelijk dat hetzelfde geldt voor andere intracellulaire levende micro-organismen, inclusief parasieten. Dit laatste is één van de verklaringen voor de hoge frequentie van MBL deficiënte genotypen in Afrika. Dit is een onderwerp van verder onderzoek.
Hoofdstuk 11 beschrijft de relatie tussen MBL genotypen en MBL serum spiegels. MBL is een acuut fase proteïne en MBL serum spiegels zijn afhankelijk van het MBL genotype. In XA/XA, XA/O en O/O genotypen zijn de MBL serum spiegels laag of afwezig en deze genotypen worden beschouwd als MBL deficiënt. Tijdens opname zijn de verschillen in MBL serum spiegels tussen MBL sufficiënte patiënten groot, maar MBL spiegels zijn weer gelijk enige tijd na ontslag uit het ziekenhuis. Dit in tegenstelling tot de spiegels in MBL deficiënte patiënten, die altijd laag of afwezig zijn. In het verleden werd MBL deficiëntie gedefinieerd als lage of afwezig MBL spiegels in de acute fase, hetgeen in onze patiëntenpopulatie uitsluitend geldt voor patiënten met een MBL deficiënt genotype. Of lage

MBL serum spiegels in MBL sufficiënte patiënten het resultaat zijn van verminderde aanmaak of verhoogd gebruik is een onderwerp van lopend onderzoek, waarin tevens gekeken wordt naar de relatie met de fagocyterende functie van MBL.

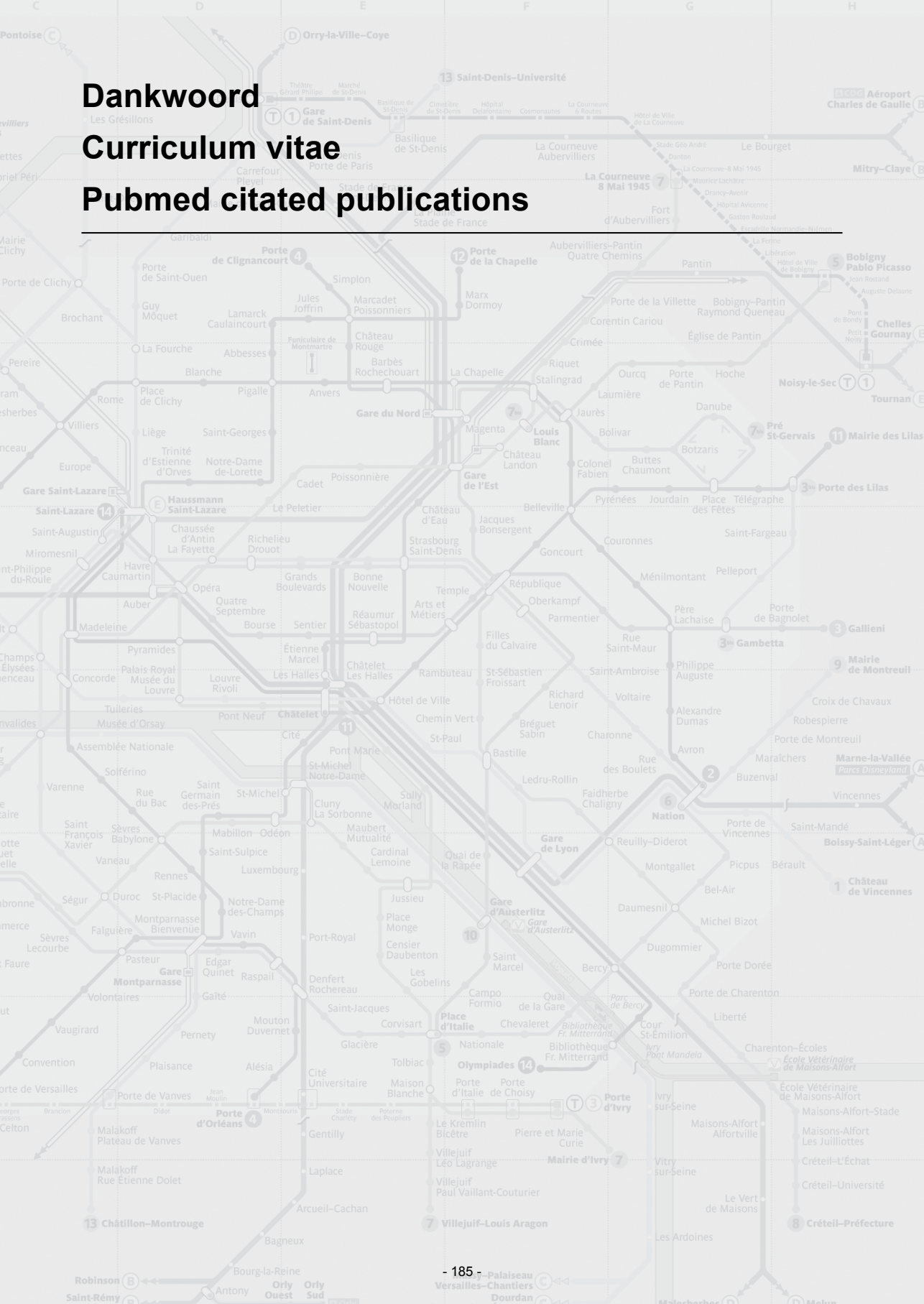
MBL deficiënte genotypen zijn gerelateerd aan een verhoogde vatbaarheid voor virale infectie tijdens pneumonie, maar niet voor pneumokokken pneumonie (Hoofdstuk 12), zoals eerder in de literatuur gemeld. Een mogelijke verklaring voor het verschil tussen deze en voorgaande bevindingen ligt in het ontwerp van de studies. Voorgaande studies waren retrospectief van opzet en patiënten werden geïncludeerd op basis van positieve bloedkweken afgenomen tijdens ziekenhuisopname, terwijl onze studie prospectief van opzet is en patiënten bevat met een pneumokokken pneumonie met en zonder positieve bloedkweek. De resultaten van deze studie geven informatie over het risico van genetische variaties in het MBL gen voor het krijgen van een pneumonie, terwijl voorgaande studies rapporteren over het risico om een bacteremie te ontwikkelen tijdens opname. De afwezige relatie tussen MBL deficiëntie en infectie met *Streptococcus pneumoniae* is conform bevindingen in experimenteel onderzoek, waarbij MBL slechts zwak aan de pneumokok bindt, maar sterk aan influenza virus. Dit laatste zou een verklaring kunnen zijn voor de overpresentatie van MBL deficiënte patiënten in de groep van patiënten met een pneumonie met een virale verwekker of co-infectie. MBL is ook een sterke *Staphylococcus aureus* binder (tevens gemeld in Hoofdstuk 12) en lopend onderzoek richt zich op populaties patiënten met stafylokokken infecties, bijvoorbeeld patiënten met een peritonitis bij continue ambulante peritoneaal dialyse, en de relatie met MBL deficiëntie (Thales-project).

Algehele beschouwing

Streptococcus pneumoniae en *Haemophilus influenzae* zijn de meest frequent geïdentificeerde verwekkers van een pneumonie. Bepaalde karakteristieken, waaronder klinische kenmerken en antibioticagebruik voor opname, kunnen het falen van identificatie van een verwekker van een pneumonie voorspellen. Door gebruik van een driestaps diagnostisch model is het mogelijk om binnen 24 uur na opname alle klinisch relevante micro-organismes te identificeren; dit model bestaat uit kweken van sputum en bloed, urine antigen testen en PCR van het sputum voor *Chlamydophila species*, *Legionella pneumophila* en *Mycoplasma pneumoniae*.
De rol van het ACE I/D polymorphisme is beperkt bij een pneumonie, maar lage ACE serum spiegels zijn wel geassocieerd met ernst van ziekte.
Binnen het humorale afweersysteem zijn talloze genetische variaties geassocieerd met vatbaarheid voor en klinisch beloop van een pneumonie veroorzaakt door een bepaalde verwekker. IL-6 en IL-10 polymorfismen zijn geassocieerd met verhoogde vatbaarheid voor het krijgen van een ernstige pneumonie of een pneumonie bij jonge patiënten. Zowel IL-6 als IL-10 serum spiegels reflecteren de ernst van inflammatie en voorspellen het klinisch beloop. Het TLR2 2477 A allel en het Fcγ-RIIa R131 genotype zijn geassocieerd met een verhoogd risico op pneumonie veroorzaakt door *Haemophilus influenzae* en het TLR5 392stop en Fcγ-RIIa R131 genotype zijn geassocieerd met verhoogd risico op (ernstige) pneumokokken pneumonie. Fcγ-RIIa H131 (wild type) en MBL sufficiënte genotypen zijn geassocieerd met een verhoogde vatbaarheid voor een pneumonie veroorzaakt door *Legionella pneumophila* en *Mycoplasma pneumoniae*. MBL genotypen bepalen MBL serum spie-

gels en MBL deficiëntie is geassocieerd met een hoger risico op virale infecties bij een pneumonie. Het antwoord op de centrale vraag van dit proefschrift – zijn variaties in de genen van het humorale afweersysteem geassocieerd met vatbaarheid voor of beloop van een pneumonie – is positief, maar vooral specifiek voor een pneumonie veroorzaakt door één micro-organisme.

Dankwoord
Curriculum vitae
Pubmed citated publications



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Saskia, dank, gewoon omdat je er bent.

Curriculum vitae

Henrik Endeman was born on February the 1st in IJsselstein, the Netherlands. He attended secondary school at the Dr. F. H. de Bruyne Lyceum in Utrecht and graduated in 1993. After secondary school he studied medicine at the University of Utrecht. During his study he was member of several faculty committees, including the faculty board with the task to develop a new medical curriculum (CRU'99), editor of a medical magazine for students and member of the JOPI foundation (Young Researchers in Pediatric Immunology). He interrupted his study for two years to become project manager within the taskforce CRU'99 and founded a consultancy agency together with drs. ing. Marco D'Agata. As an intern, he worked 4 months at the Neonatology Unit (NICU) of the Hopital Edouard Herriot, Lyon, France and 4 months at the Intensive Care Unit (ICU) of the Diakonessenhuis in Utrecht. During this internship he performed research on the subject of prolonged mechanical ventilation.

In 2001 he started the training in Internal Medicine at the St. Antonius Ziekenhuis, Nieuwegein (head of department: dr. Huub Haanen and prof. dr. Douwe Biesma). After a 6 month course in laboratory medicine in the Central Laboratory of Sanquin, Amsterdam (head of department: prof. dr. Dirk Roos), he started the Triple-P study, which is the basis for this thesis. He finished his training in Internal Medicine in 2001 in the Onze Lieve Vrouwe Gasthuis, Amsterdam, and continued with the training in Intensive Care Medicine at the same hospital (head of department: prof. dr. Durk Zandstra) and the Academic Medical Centre in Amsterdam. During this time he participated in studies on the use of tobramycin as a part of selective decontamination of the digestive tract. He finished the training in Intensive Care Medicine in 2008, in the same year he successfully passed the European Intensive Care Medicine examinations. Since August 2008 Henrik Endeman is working as an intensivist in the Diakonessenhuis, Utrecht, and is still involved in research projects at the St. Antonius Ziekenhuis and the Onze Lieve Vrouwe Gasthuis.

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