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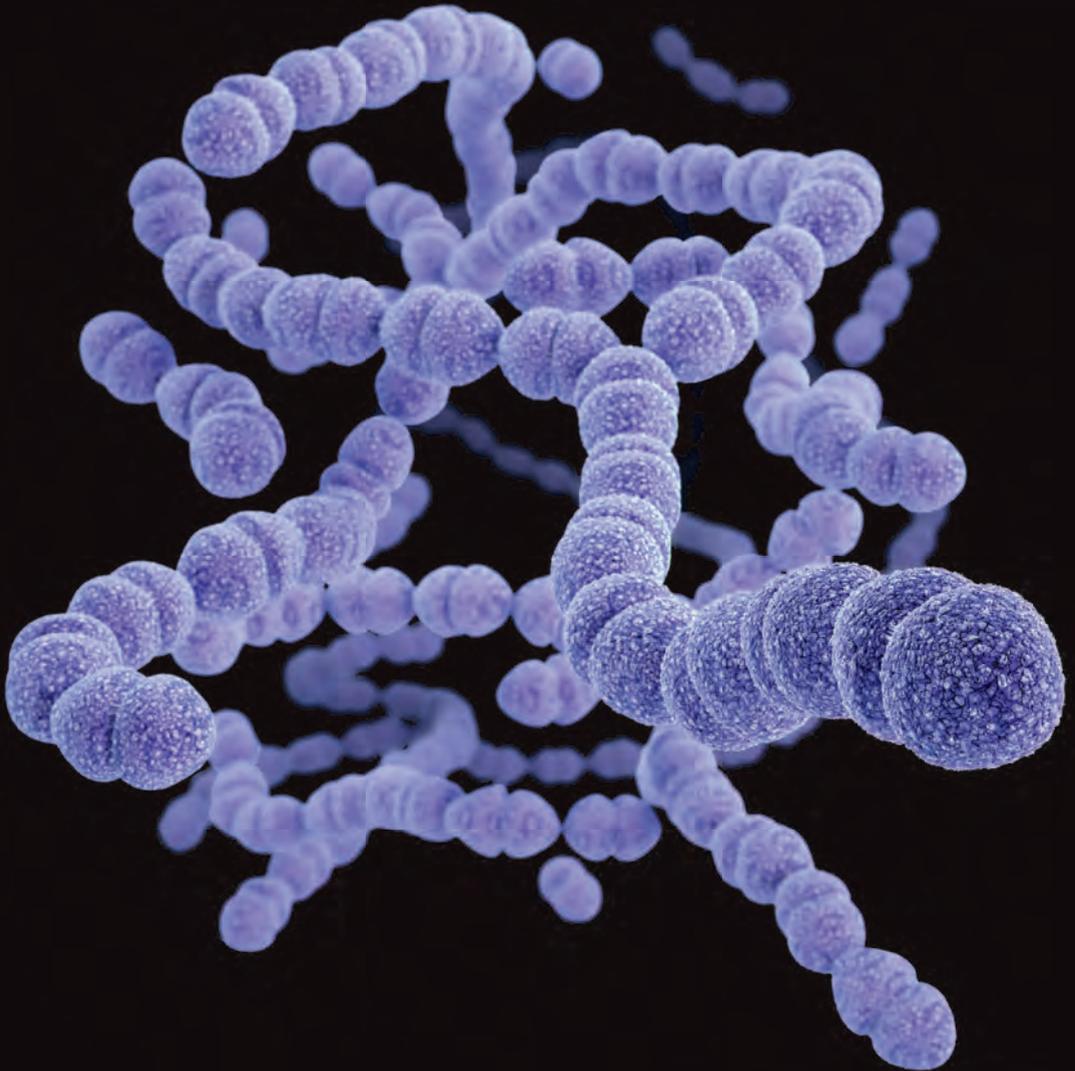
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# **PATHOPHYSIOLOGY OF PNEUMOCOCCAL MENINGITIS**



**Madelijn Geldhoff**



# Pathophysiology of pneumococcal meningitis

Madelijn Geldhoff

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# Pathophysiology of pneumococcal meningitis

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ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

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*Voor Miep, Brecht en ...*



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# INTRODUCTION

Bacterial meningitis is a serious infectious disease, involving the membranes surrounding the brain and spinal cord, and the subarachnoid space. In the Netherlands most common causative agents are *Streptococcus pneumoniae* (72%) and *Neisseria meningitidis* (11%).<sup>1</sup> The incidence of pneumococcal meningitis in the Netherlands is 0.7 per 100,000, and has rapidly declined over the last decade as a result of herd immunity established by the introduction of a pneumococcal conjugate vaccine in the national childhood immunization programme.<sup>2</sup> However, mortality and morbidity of pneumococcal meningitis remain high.<sup>3</sup>

*S. pneumoniae* was first isolated coincidentally by Pasteur and Sternberg in 1881 from saliva of a patient.<sup>4,5</sup> In 1883 the pneumococcus was first associated with pneumonia, and in 1884 the Gram stain was developed which led to more specific identification of the pneumococcus and its association with other infectious diseases such as otitis and meningitis.<sup>6</sup> In the beginning of the 20<sup>th</sup> century the chemical composition of the pneumococcus was revealed and different pneumococcal capsular polysaccharides were identified and their role in virulence was described.<sup>6</sup> Studies showed that serum from infected individuals protected from disease and in 1911 the first pneumococcal vaccine was produced from killed pneumococci and tested in humans by Wright et al.<sup>6</sup> By 1940 over 85 different pneumococcal serotypes were identified and the role of the capsular polysaccharides in recognition by the host immune system was established.<sup>6</sup> It was shown that opsonization by complement and capsular type specific antibodies leads to phagocytosis and killing of the bacteria by submucosal macrophages.<sup>7</sup>

The pneumococcus is a human commensal bacterium that resides at the nasopharyngeal mucosa in up to 5-90% of the population and is transferred between people mainly via coughing and sneezing.<sup>8</sup> Nasopharyngeal carriage usually results in protection against infection with the specific serotype.<sup>9</sup> Although in the majority of cases *S. pneumoniae* colonization is asymptomatic, it may proceed to infection in some individuals.<sup>10</sup> Most common pneumococcal infections are upper respiratory tract infections, otitis, sinusitis and pneumonia.<sup>11</sup> Invasive disease such as sepsis and meningitis are less common but more serious diseases with high mortality and morbidity.<sup>1,12</sup> The highest incidence of pneumococcal disease occurs in children under the age of two and the elderly above 65 years of age.<sup>8</sup>

Before the discovery of antibiotics in 1929 by Fleming pneumococcal meningitis was an invariably fatal disease.<sup>13</sup> Since the general introduction of penicillin in the 1940's the mortality of pneumococcal meningitis has declined to 30% and has remained steady since then for a long period despite advances in supportive medical care.<sup>1</sup> The only adjuvant treatment that has proven to be effective in adult patients with pneumococcal meningitis to date is dexamethasone.<sup>14</sup> The introduction of dexamethasone as an adjuvant treatment next to antibiotics, has decreased mortality of pneumococcal meningitis from 30 to 20% and unfavorable outcome from 50 to 39%.<sup>1</sup> However, still up to 40% of the patients surviving pneumococcal meningitis suffer from neurologic sequelae, including hearing loss, aphasia, paresis, cranial nerve palsies and cognitive impairment.<sup>3,15</sup> Pathologic findings on brain imaging occur in up to 56% and most commonly include sinusitis or otitis, ischemic

stroke, cerebral edema, cerebritis and hydrocephalus.<sup>16</sup> Several clinical risk factors for unfavorable outcome of pneumococcal meningitis have been identified, including age, immunocompromised state (e.g., patients on immunosuppressive drugs, asplenia, diabetes mellitus, alcoholism or HIV infection), systemic infection, and parameters indicating poorly controlled cerebrospinal fluid (CSF) infection (CSF white blood cell count <1000 / mm<sup>3</sup>, high CSF protein count, low CSF-blood glucose ratio).<sup>16</sup> Genetic risk factors include deficiencies in the innate immune response, which lead to overwhelming pneumococcal infections in these patients and is associated with high morbidity and mortality.<sup>17</sup> As opposed to immunodeficiency and uncontrolled infection, the host immune response itself may lead to host tissue damage and unfavorable outcome.<sup>18</sup> Several components of the host immune response associated with brain damage and adverse disease outcome have been described in experimental animals. Previous studies showed that a genetic variation in complement component 5 and increased CSF levels of complement factors C5a and the terminal complement complex (C5b-9) are associated with unfavorable outcome in patients with bacterial meningitis.<sup>19</sup> However, little is known about the host inflammatory response with respect to disease outcome in humans. The aim of this thesis is to gain more insight in the pathophysiology of pneumococcal meningitis and to identify new targets for potential adjuvant treatments in the future.

In chapter 2 the pathophysiology of pneumococcal meningitis is reviewed. We discuss the different routes of infection, the host innate and adaptive immune response, brain damage described in humans and different animal models and potential targets for adjunctive therapy. In chapter 3 we describe the development and validation of an experimental mouse model for pneumococcal meningitis. We describe the different features of the immune response and pathophysiology. This animal model is used to study the role of the inflammasome pathway in the pathogenesis of pneumococcal meningitis in chapter 4. In chapter 5 we describe the association of a single-nucleotide polymorphism in two genes that are involved in the inflammasome-signaling pathway with unfavorable outcome in patients with pneumococcal meningitis. In chapter 6 we show that pneumococcal virulence also plays a role in the outcome of pneumococcal meningitis in patients. The absence of a pneumococcal arginine synthetase system is associated with favorable outcome in patients. In chapter 7 we report an explorative study in which we measured a large set of inflammatory mediators in the CSF of patients with pneumococcal meningitis. In chapter 8 we discuss our results with regard to other studies, we address any methodological shortcomings and give directions for future research.

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# 2

## Pathogenesis and pathophysiology of pneumococcal meningitis

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## Abstract

Pneumococcal meningitis continues to be associated with high rates of mortality and long-term neurological sequelae. The most common route of infection starts by nasopharyngeal colonization by *Streptococcus pneumoniae*, which must avoid mucosal entrapment and evade the host immune system after local activation. During invasive disease, pneumococcal epithelial adhesion is followed by bloodstream invasion and activation of the complement and coagulation systems. The release of inflammatory mediators facilitates pneumococcal crossing of the blood-brain barrier into the brain, where the bacteria multiply freely and trigger activation of circulating antigen-presenting cells and resident microglial cells. The resulting massive inflammation leads to further neutrophil recruitment and inflammation, resulting in the well-known features of bacterial meningitis, including cerebrospinal fluid pleocytosis, cochlear damage, cerebral edema, hydrocephalus, and cerebrovascular complications. Experimental animal models continue to further our understanding of the pathophysiology of pneumococcal meningitis and provide the platform for the development of new adjuvant treatments and antimicrobial therapy. This review discusses the most recent views on the pathophysiology of pneumococcal meningitis, as well as potential targets for (adjunctive) therapy.

## Introduction

Community-acquired bacterial meningitis continues to exact a heavy toll, even in developed countries, despite the implementation of childhood vaccination programs and effective antimicrobial agents.<sup>1,2</sup> The most common etiologic agents are *Streptococcus pneumoniae* and *Neisseria meningitidis*, with the first being responsible for two-thirds of cases in Europe and the United States.<sup>3-5</sup> Today, despite advances in medical care, mortality from pneumococcal meningitis ranges from 16 to 37%, and neurological sequelae, including hearing loss, focal neurological deficits, and cognitive impairment, are estimated to occur in 30 to 52% of surviving patients.<sup>5-9</sup>

During past decades, experimental animal models have shown that the outcome of bacterial meningitis is related to the severity of inflammation in the subarachnoid space and that the outcome can be improved by modulation of the inflammatory response, e.g., with dexamethasone.<sup>10</sup> Many randomized clinical trials of dexamethasone in bacterial meningitis have been performed, but the results remain ambiguous.<sup>4,11-15</sup> An individual patient data meta-analysis of 5 large recent trials showed no effect of dexamethasone.<sup>16</sup> However, a prospective cohort study showed a decrease in mortality from 30 to 20% in adults with pneumococcal meningitis after successful nationwide implementation of dexamethasone in The Netherlands.<sup>17</sup> Nevertheless, new adjunctive therapies are needed to improve the prognosis of bacterial meningitis.

Previously, we reviewed the epidemiology, diagnosis, and antimicrobial treatment of acute bacterial meningitis.<sup>4</sup> In the current review, we focus on current understandings of the pathophysiology and pathogenic mechanisms associated with pneumococcal meningitis. Finally, we discuss targets for future therapeutic strategies.

## Colonization

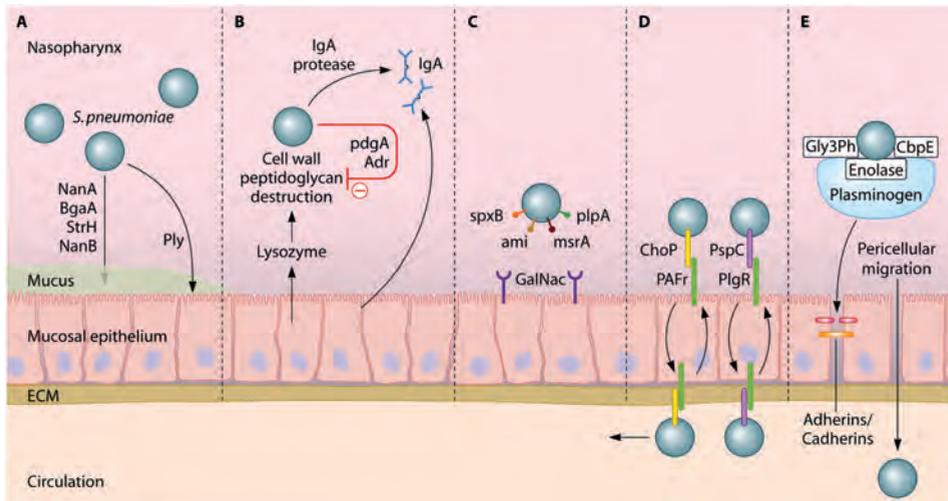
### Mucosal colonization

The human nasopharynx is the main reservoir for *S. pneumoniae*, where it usually leads to asymptomatic colonization. Carriage rates of *S. pneumoniae* are highest among young children (37%) and may rise to up to 58% in crowded situations such as day care centers.<sup>18</sup> In adults, crowding may also lead to increased carriage rates, specifically in hospitals, long-term care facilities, shelters, and prisons, where carriage rates of up to 40% have been reported, compared to 4% in the general adult population.<sup>19-21</sup> The bacterium is transferred between people mainly by coughing and sneezing. During colonization, adherence, nutrition, and replication are the pneumococcus' main priorities. To reach these objectives, the pneumococcus is confronted with the host's natural barriers at the respiratory mucosa, the host's immune system, and other pathogens colonizing the same niche.

## Natural barrier evasion

Two important natural barriers preventing pneumococci from binding to the respiratory mucosal surface are the respiratory mucus and lysozyme.<sup>22-24</sup> The pneumococcus has evolved several strategies to overcome these barriers and reach the respiratory epithelial cell layer.

Mucus entrapment and subsequent clearing may be prevented by the pneumococcus by three ways. First, the capsule of the pneumococcus repulses the sialic acid residues of mucus by its negative charge, thereby decreasing the likelihood of entrapment.<sup>23</sup> Second, the pneumococcus expresses several exoglycosidases, including neuraminidase A (NanA), beta-galactosidase A (BgaA), beta-*N*-acetylglucosaminidase (StrH), and neuraminidase B (NanB), which are capable of deglycosylating mucus glycoconjugates, thereby decreasing mucus viscosity and preventing mucus entrapment.<sup>25-27</sup> Third, pneumolysin (Ply), a pore-forming toxin, decreases epithelial cell ciliary beating, thereby enabling the pneumococcus to bind to epithelial cells without being removed with the mucus (Figure 1A).<sup>28,29</sup>



**Figure 1:** (A) Mucus breakdown. *S. pneumoniae* colonization of the nasopharynx is facilitated by mucus degradation by the enzymes NanA, BgaA, StrH, and NanB. Ply decreases epithelial cell ciliary beating, enhancing bacterial adherence. (B) Evasion of proteolytic enzymes. Pneumococcal cell wall peptidoglycans may be destroyed by lysozyme. PdgA and ADR deacetylate pneumococcal cell surface peptidoglycan molecules, rendering them resistant to lysozyme. (C) Epithelial cell binding. *S. pneumoniae* binds host GalNac by using SpxB, Smi, MsrA, and PlpA. (D) Intracellular translocation. By binding the pIgR with PspC (or PAF receptor [PAFr] with ChoP), pneumococci can use the pIgR or PAF receptor recycling pathway to be transported through the epithelial cell layer. (E) Inter- and pericellular translocation. Plasminogen bound by Gly3Ph, CbpE, and enolase enhances epithelial cell binding and degrades interepithelial adherens junctions, allowing pericellular migration.

Lysozyme is a muramidase which cleaves peptidoglycan, a polymer of sugars and amino acids present in the cell wall of many pathogens, including *S. pneumoniae*.<sup>30</sup> Acetylated peptidoglycan molecules of the pneumococcal cell wall (PCW) are specifically prone to lysozyme destruction. The pneumococcus expresses two enzymes, peptidoglycan

*N*-acetylglucosamine-deacetylase A (PdG<sub>A</sub>) and an *O*-acetyltransferase (Adr), which are able to deacetylate peptidoglycan molecules on the pneumococcal surface, rendering the bacterium resistant to lysozyme (Fig. 1B).<sup>30-32</sup> Both enzymes have been shown to be important during colonization, as PdG<sub>A</sub> or Adr knockout pneumococci are more prone to exogenous lysozyme and are outcompeted by wild-type (WT) pneumococci in an intranasal model of pneumococcal colonization.<sup>30</sup>

### Host mucosal immune system

At the nasopharyngeal mucosal site, the pneumococcus is targeted by components of the host innate immune system, such as secretory IgA (sIgA), lactoferrin, and components of the complement system.<sup>33-36</sup>

Soluble IgA interferes with binding of the pneumococcus to the nasopharyngeal mucosa and facilitates opsonization of bacteria, which enables phagocytosis by antigen-presenting cells (APCs) and neutrophils.<sup>37-39</sup> Pneumococci have several methods to limit opsonization by sIgA. First, the capsule itself prevents binding of sIgA.<sup>40</sup> Second, capsule-bound IgA is cleaved by a pneumococcal IgA1 protease. This protease cleaves sIgA at the hinge region, inhibiting IgA-mediated opsonization and promoting binding to the respiratory mucosa.<sup>41,42</sup> The remaining Fab fragment of sIgA binds to the PCW, thereby exposing choline-binding proteins (Cbps) and decreasing the negative charge of the capsule, which also facilitates bacterial adhesion to the epithelial cell (Fig. 1B).<sup>42</sup>

Lactoferrin is an iron scavenger present in multiple human body fluids, including saliva and nasal secretions.<sup>43</sup> Lactoferrin acts bacteriostatically by depleting iron necessary for bacterial metabolism. Unbound lactoferrin (apolactoferrin) also has direct bactericidal properties, independent of iron scavenging, toward various pathogens, including *S. pneumoniae*.<sup>35,44,45</sup> The mechanism by which apolactoferrin destroys bacteria is not completely clear, but it appears to disrupt the bacterial cell, leading to cell lysis.<sup>46</sup> Lactoferrin is also present in neutrophils and may enhance bacterial phagocytosis and killing.<sup>47</sup> The pneumococcus prevents apolactoferrin-mediated killing by the expression of pneumococcal surface protein A (PspA), a choline-binding protein expressed on the outer surface of the pneumococcal cell. PspA binds human apolactoferrin at its active site, thereby inhibiting apolactoferrin-mediated bacterial killing.<sup>35</sup>

A third, important component of the mucosal innate immune system is the complement cascade. Activation of the complement pathway results in cleavage of several complement factors, leading to bacterial opsonization and phagocytosis, leukocyte recruitment, and the assembly of a membrane attack complex (MAC) which forms pores in the pathogen's membrane, inducing cell lysis.<sup>48</sup> Complement plays an important role in the immune response against *S. pneumoniae*, since mice as well as humans with complement deficiencies are more susceptible to the transition of pneumococcal colonization to invasive disease.<sup>33,34,49</sup>

C-reactive protein (CRP) serves as an important innate immune defense mechanism of the respiratory tract.<sup>50</sup> CRP is a protein produced by the liver in the acute phase of

an infection.<sup>48</sup> CRP binds to phosphorylcholine on apoptotic cells and several bacteria, including the pneumococcus.<sup>51,52</sup> Through binding on the bacterial cell surface, CRP can activate the classical complement pathway through complement factor 1q (C1q).<sup>53</sup> Subsequent opsonophagocytosis by the complement system leads to more effective phagocytosis by macrophages. In addition, CRP can bind the Fcγ receptor (FcγR) on macrophages and dendritic cells, thereby enhancing phagocytosis and macrophage cytokine production.<sup>54-56</sup>

The complement cascade is activated in three ways: the classical complement pathway, the alternative complement pathway, and the lectin-induced complement pathway. The classical complement pathway is characteristically activated by antibody-antigen complexes. Natural IgM, a part of which is directed against pneumococcal C polysaccharides (teichoic acid), contributes to the activation of the classical pathway.<sup>57</sup> However, the classical pathway may also be activated through other mechanisms, such as by the binding of acute-phase proteins such as CRP to the pneumococcal surface and subsequent binding of complement component C1q, direct binding of C1q to the bacterium, and binding of C1q to the C-type lectin SIGN-R1.<sup>48,58</sup> When C1q was depleted from human serum, *in vitro* opsonophagocytosis of *S. pneumoniae* was severely affected.<sup>59</sup> In addition, C1q-deficient mice showed a severely impaired immune response and worse outcomes in an experimental model of pneumococcal meningitis.<sup>60</sup> Furthermore, mice deficient in the pattern recognition receptor SIGN-R1 had reduced activation of the classical complement pathway.<sup>58</sup> In this study, C1q was directly activated upon activation of SIGN-R1 by pneumococcal polysaccharides in the spleen, leading to activation of the classical complement cascade and complement component C3 activation, with subsequent pneumococcal opsonization.<sup>58</sup> SIGN-R1 is highly abundant on cells of the splenic red pulp and is an important factor in the spleen's function to control invasive pneumococcal disease. Another study showed that splenic macrophages of SIGN-R1 knockout mice were unable to activate splenic B cells to produce pneumococcus-specific IgM.<sup>61</sup> Therefore, splenic SIGN-R1-mediated activation of B cells may explain, at least partially, the susceptibility of splenectomized patients to invasive pneumococcal disease.

Activation of C1q by the classical or mannose-binding lectin (MBL) pathway leads to cleavage of complement component C2. In a Swedish cohort, 40 patients with a homozygous C2 deficiency due to a deletion in the C2 gene were described.<sup>62</sup> Invasive infections, mainly pneumococcal infections, were found in 23 (58%) of these patients.<sup>62</sup>

The alternative pathway is also activated during infection with *S. pneumoniae* and occurs by the direct binding of complement component C3 to the pneumococcal surface.<sup>63</sup> The importance of the alternative pathway in pneumococcal opsonization was shown in mice made deficient in factor D, a peptidase involved in activation of the alternative pathway.<sup>64</sup> Opsonophagocytosis of *S. pneumoniae* was delayed in factor D-deficient mice compared to wild-type mice, indicating an important role for this complement pathway in the early phase of infection.<sup>64</sup> In line with this, a recent study showed that mice deficient

in complement factor B, another peptidase involved in activation of the alternative complement pathway were more susceptible to pneumococcal otitis media.<sup>65</sup>

The lectin-induced complement pathway appears to be less important in pneumococcal disease than the classical and alternative pathways. Polymorphisms in MBL, one of the most important activators of the lectin complement pathway, were not associated with increased risk of pneumococcal invasive disease in a genetic association study.<sup>66</sup> A larger cohort showed a significant increase in risk for pneumococcal invasive disease, with three codon variants in the MBL locus.<sup>67</sup> In a third study, 140 patients with invasive pneumococcal disease, defined by positive blood culture for *S. pneumoniae*, were assessed for three structural variant MBL alleles and one promoter allele.<sup>68</sup> In this study, no association was found between susceptibility or outcome of invasive pneumococcal disease and any of the structural MBL variants or promoter alleles. In a subgroup analysis of the 22 patients in the cohort with pneumococcal invasive disease and meningitis, there was no association between susceptibility or outcome and the MBL genotype.<sup>68</sup> However, a meta-analysis combining the results of the above three studies demonstrated an association between susceptibility to invasive pneumococcal disease and homozygosity for one of the three structural variants in the MBL gene, with an odds ratio (OR) of 2.57 (95% confidence interval [CI], 1.38 to 4.80).<sup>69</sup> In a cohort of 57 HIV-positive patients, an increased risk for invasive pneumococcal disease was found to be associated neither with MBL polymorphisms nor with polymorphisms in the downstream molecule MBL-associated serine protease 2 (MASP-2).<sup>70</sup> One genetic association study has been performed regarding outcome and MBL genotypes. This study included only 60 patients with community-acquired pneumococcal pneumonia and did not detect an association between MBL genotype and outcome.<sup>71</sup> Experimental studies showed weak to no binding of MBL to *S. pneumoniae* compared to other bacteria.<sup>72,73</sup> Another experimental study showed that although MBL bound to *S. pneumoniae*, it did not increase opsonophagocytosis, and that complement activation by the classical pathway was much more important.<sup>74</sup>

Another group of proteins that can activate the lectin-induced complement pathway are ficolins. Two ficolin variants, H-ficolin and L-ficolin, have been studied for the capability of binding to *S. pneumoniae*; only L-ficolin was found to bind some of the pneumococcal strains tested.<sup>73</sup> However, no frequency differences were found for polymorphisms in L-ficolin among 290 patients with invasive pneumococcal disease compared to 720 controls from a similar population.<sup>75</sup>

The pneumococcus has evolved several strategies to limit complement-mediated opsonophagocytosis. The pneumococcal capsule plays a central role by limiting the amount of complement deposited on the pneumococcal surface and impeding the access to cell-bound complement.<sup>76</sup> Furthermore, pneumolysin has been shown to decrease complement opsonization of the pneumococcal cell.<sup>77</sup> This is thought to result from the consumption of complement factors by released pneumolysin. In addition, several other pneumococcal outer surface proteins have been shown to affect complement deposition on

the pneumococcus, including pneumococcal surface protein C (PspC), PspA, PsaA, and PhpA.<sup>36,77-83</sup>

PspC, also referred to as CbpA or SpsA, a choline-binding protein attached to the cell wall, is able to bind complement component C3b, thereby preventing opsonization.<sup>36,78,79,81</sup> Furthermore, PspC binds human factor H, a factor which inhibits activation of two complement components of the alternative and lectin pathways. By binding and activating factor H, the pneumococcus locally blocks the unfolding of these two complement pathways.<sup>81,84-86</sup> In addition, PspC binds the complement inhibitor C4b-binding protein, which blocks activation of the classical complement pathway.<sup>87</sup> PspA has been shown to interfere with the binding of complement component C3 on the bacterial surface, thereby inhibiting complement-mediated opsonization.<sup>77,80,82</sup> PhpA is a pneumococcal surface protein with C3-degrading properties.<sup>83</sup> Since activation of the complement cascade is crucial in the defense against pneumococcal invasive disease, pneumococcal complement binding proteins are important targets for vaccine development.<sup>88-91</sup>

### Binding to epithelium

The pneumococcal capsule is advantageous in circumventing the host barriers and reaching the respiratory mucosa but covers PCW binding sites for epithelial cell binding. The pneumococcus adjusts its binding properties to its environment through a process called phase variation.<sup>92-94</sup> In this process, the amount of polysaccharide in the capsule varies from an opaque (thick capsule) to a transparent (thin capsule) phase, either covering or exposing binding sites on the pneumococcal surface.<sup>92</sup> During colonization, the thick capsule prevents mucus entrapment as well as immunoglobulin and complement binding, thereby preventing opsonophagocytosis.<sup>23,95-97</sup> Once the pneumococcus has reached the nasopharyngeal epithelium, the transparent phase becomes prominent, unveiling several adhesion molecules for binding to the host epithelium.<sup>92,94</sup>

At the host respiratory epithelium, the pneumococcus binds to glycoconjugates expressed on the epithelial cells of the respiratory mucosa (e.g., N-acetyl-D-galactosamine [GalNac]). Pneumococcal binding molecules interacting with the host glycoconjugates remain elusive. However, several bacterial genes involved in GalNac binding have been identified, including *spxB*, *ami*, *msrA*, and *plpA* (Fig. 1C).<sup>98-100</sup> Their gene products are involved either directly in binding of glycoconjugates or indirectly by inducing upregulation of their binding molecules on the epithelial lining.<sup>98,101-104</sup> Binding of the pneumococcus to GalNac is promoted by NanA, a pneumococcal glycosidase that separates sialic acid from mucin, glycolipids, glycoproteins, and oligosaccharides, thereby enhancing the expression of N-acetylglucosamine binding sites on host epithelial cells.<sup>27,105</sup> Cleaved sialic acid residues serve as a carbohydrate source for bacterial metabolism.<sup>25,26</sup>

Pneumococcal binding is further enhanced by hydrophobic and electrostatic forces, binding of pneumococcal phosphorylcholine to the platelet activating factor (PAF) receptor, and binding of pneumococcal surface protein C (PspC) to the polymeric

immunoglobulin (pIgR) receptor, all facilitating epithelial cell transcytosis (see Bloodstream Survival).<sup>37,106,107</sup> Pneumococci also display pili on their surfaces, facilitating adherence to human buccal cells in the nasopharynx; however, which components of the respiratory mucosa interact with the pili are unknown.<sup>108-110</sup>

### Co-colonization

The nasopharynx may be colonized by up to 700 different microbial species, including residential flora, transient colonizing microbes, and pathogenic species.<sup>111,112</sup> Microbial survival is therefore dependent on cooperative and competitive strategies, several of which were recently described in the context of pneumococcal infection.<sup>113,114</sup> Pneumococcal intermicrobial interactions include secondary invasive disease following viral infection, prior innate immunity activation following exposure to another pathogen, and the sharing of virulence/survival factors between pneumococcal serotypes.<sup>115</sup>

Viral infection and subsequent bacterial infection have been investigated extensively.<sup>115-117</sup> Prior exposure to influenza virus has been associated with secondary invasive pneumococcal disease.<sup>118,119</sup> The importance of preexposure to influenza virus was recently underlined during the H1N1 pandemic, in which a third of fatal H1N1 cases exhibited evidence of concurrent bacterial pneumonia.<sup>120</sup> The underlying pathogenesis of enhanced susceptibility to invasive pneumococcal disease after influenza virus infection remains unclear but might be related to an altered expression of adhesion molecules. Prior exposure to viral infection has been demonstrated to increase the expression of epithelial cell adhesion molecules both *in vitro* and *in vivo*.<sup>121</sup> The exposure of adhesion molecules on the epithelial lining is further aided by influenza virus neuraminidase (NA), which cleaves terminal sialic acid residues, thereby facilitating pneumococcal binding after viral exposure.<sup>122</sup> In mice, pneumococcal binding was reduced when NA was blocked pharmacologically or when either the pneumococcus or influenza virus was mutated to be NA deficient.<sup>123</sup> Of particular interest has been the PAF receptor, which may be used by pneumococci for adherence to and transcytosis of the epithelium. Though the PAF receptor is upregulated following viral exposure, murine PAF receptor knockout studies yielded conflicting results regarding the contribution of PAF receptor to pneumococcal adherence and subsequent invasion.<sup>124-126</sup> These conflicting results might be explained by variations in pneumococcal serotype, dosing, and timing of coinfection. There are alternative explanations to PAF receptor upregulation for the association of viral and bacterial infections, including mechanical lung epithelium damage, overall impaired pulmonary function, and an altered immune response to secondary infection following viral exposure.<sup>115</sup> *Ex vivo* studies in which the tracheal epithelium was severely damaged following viral infection did not show increased binding of *S. pneumoniae* but showed a decreased mucociliary velocity leading to a higher local bacterial burden after secondary infection.<sup>127</sup>

Nasopharyngeal interactions between cocolonizing bacteria can lead to growth inhibition, synergism, and exchange of genetic material. Epidemiologic data suggested a

negative association between nasopharyngeal colonization of *Staphylococcus aureus* and *S. pneumoniae*.<sup>128,129</sup> *In vitro* studies suggested that *S. aureus* killing was the result of pneumococcal H<sub>2</sub>O<sub>2</sub> production, but this effect has not been reproduced invariably *in vivo*.<sup>114,130</sup> Bacteria may also compete or synergize in the nasopharynx by using the host response. Cocolonization of *S. pneumoniae* and *Haemophilus influenzae* led to rapid neutrophil-mediated clearance of *S. pneumoniae*.<sup>131</sup> *In vitro* studies revealed that cell components of *H. influenzae* specifically stimulated the complement-dependent phagocytosis of *S. pneumoniae*; depletion of either complement or neutrophils abolished this competitive phenomenon.<sup>131</sup>

Finally, multiple pneumococcal strains may cocolonize the nasopharynx, usually leading to intraspecies competition and competitive outgrowth of a single strain.<sup>132,133</sup> One proposed mechanism for this intraspecies competition involves the use of bacteriocins, so-called pneumocins in pneumococci, which are small peptides capable of killing bacteria of the same or closely related species.<sup>134</sup> Additionally, *S. pneumoniae* is naturally able to integrate DNA from killed and closely related pathogens into its own genome, thus gaining a competitive advantage.<sup>132</sup> In *in vitro* cocultures, pneumococci that were made bacteriocin deficient were rapidly outcompeted by parent strains or pneumococci of other serotypes.<sup>113</sup>

## Invasive disease

### Patients at risk

Invasive pneumococcal disease may take place when two situations coincide: first, the host is colonized with a pneumococcal strain that it has not yet established immunity to, and second, an alteration of the natural barriers or host immune system has occurred.<sup>135,136</sup> Invasive pneumococcal disease is seen during the extremes of age (less than 2 or more than 50 years of age); in patients with underlying conditions, such as splenectomy or asplenic states, sickle cell disease, multiple myeloma, hypogammaglobulinemia, alcoholism, chronic liver or kidney disease, malignancy, malnutrition, Wiskott-Aldrich syndrome, thalassemia major, diabetes mellitus, and basilar skull fracture with leakage of cerebrospinal fluid (CSF); and in children with cochlear implants.<sup>1,2,137-148</sup> The use of immunosuppressive drugs, a history of splenectomy, or the presence of diabetes mellitus, alcoholism, or infection with HIV is found in 20% of adults with pneumococcal meningitis.<sup>144,147</sup> Furthermore, damage to the naso- and oropharyngeal mucosae may be elicited by local pneumococcal infection, such as sinusitis or otitis, by viral respiratory infections (specifically by influenza virus [see “Cocolonization”), by smoking, or by allergy.<sup>9,149-151</sup>

### Invading host endothelial and epithelial cells

Pneumococci are relatively ineffective at invading host endothelial and epithelial cells. However, pressures of the host natural barriers, cocolonization of other microorganisms,

and an activated innate immune response drive pathogens to develop new strategies. Epithelial endo- and transcytosis is an important strategy of invasion and also allows intraepithelial bacterial reservoirs and subsequent recolonization of the nasopharynx. Two mechanisms of epithelial transmigration by *S. pneumoniae* have been described (Fig. 1D). First, pneumococcal phosphorylcholine (ChoP) may bind to the PAF receptor on activated epithelial and endothelial cells.<sup>106</sup> ChoP is a component of cell wall-associated acids and lipoteichoic acids (LTAs) on the surfaces of transparent pneumococci.<sup>152</sup> By binding the PAF receptor, the pneumococcus may enter the PAF receptor recycling pathway, which transports the bacterium to the basal membrane of the host epithelial cell, which may lead to invasive disease.<sup>106,153</sup> Intranasal challenge of mice deficient in the PAF receptor resulted in reduced rates of pneumococcal colonization, pneumonia, and invasive disease.<sup>125</sup>

A second mechanism involves the binding of the pneumococcal choline-binding protein PspC (also known as CbpA or SpsA) to the extracellular portion of epithelial pIgR, referred to as “secretory component”.<sup>37,107</sup> Following attachment, the pneumococcus uses the pIgR recycling pathway, analogous to the PAF receptor pathway, to be transported between the apical and basal membranes of the epithelial cell.<sup>37,154</sup> Pneumococcal expression of PspC has been shown to be an important factor for colonization and invasive disease, although its effect on virulence may vary between pneumococcal strains.<sup>79,154-157</sup> The PspC binding of pIg receptor is observed only in humans, not in mice, rats, or rabbits.<sup>37</sup> In addition, PspC also binds sialic acid and lacto-*N*-neotetraose on respiratory epithelial cells, further facilitating colonization.<sup>157</sup> The level of pIg receptor directly correlates with the degree of pneumococcal attachment and epithelial invasion.<sup>154</sup> pIg receptors are expressed in a decreasing gradient from the upper to the lower respiratory tract, while the opposite pattern is observed for the PAF receptor.<sup>154,158</sup> Therefore, it has been suggested that where pIg receptor serves mainly as a pneumococcal receptor in the nasopharynx, the PAF receptor acts as a ligand for attachment and invasion of the pulmonary epithelium.<sup>154</sup>

Inter- or pericellular migration is another mechanism by which bacteria may cross epithelial or endothelial cell layers (Fig. 1E).<sup>159</sup> Plasminogen, bound by the pneumococcal receptors enolase, Gly3Ph, and CbpE, plays a central role in this process and has been shown to serve two purposes.<sup>160-162</sup> First, plasminogen increases adhesion of pneumococci to the epithelial surface.<sup>163</sup> Second, bound plasmin is able to cleave proteins involved in the intercellular adherens junctions, which bind epithelial cells together to form a mechanical barrier to underlying tissues.<sup>163</sup> This disruption is mediated by the degradation of cadherin, an essential component of interepithelial adherens junctions.<sup>163</sup> Murine pneumococcal nasopharyngeal colonization studies demonstrated that epithelial barrier function was diminished through the downregulation of cadherins in a Toll-like receptor (TLR)-dependent manner.<sup>164</sup> Third, epithelial permeability is also modulated by the innate immune system in a transforming growth factor beta (TGF- $\beta$ )-dependent manner, possibly to allow for adequate migration of immune cells and inflammatory mediators

into infected areas.<sup>165</sup> Thus, the breakdown of the tight junctions, though necessary for an adequate immune response, may allow for pneumococcal access to the basal membrane and subsequent invasive disease.

### **Extracellular matrix**

At the basal side of the epithelium or endothelium lies the basement membrane, which is comprised mainly of a network of collagen type I, laminin, and proteoglycans.<sup>166</sup> Like many bacteria, pneumococci use hyaluronan lyase to degrade major components of the extracellular matrix (ECM), hyaluronan, and certain chondroitins, thereby facilitating invasive disease.<sup>167</sup> The importance of hyaluronan lyase for the development of invasive pneumococcal disease was demonstrated in mice, as intranasally administered hyaluronidase adjuvant enhanced the development of invasive disease after an otherwise noninvasive intranasal inoculation of pneumococci.<sup>168</sup> Moreover, pneumococci isolated from patients with pneumococcal meningitis expressed higher levels of hyaluronidase than pneumococci isolated from asymptomatic carriers.<sup>169</sup>

Fibronectin, a large multidomain ECM glycoprotein, is found in nearly every human tissue environment that the pneumococcus is likely to encounter and is bound by several pneumococcal adhesins, among which the most important are the pneumococcal adhesion and virulence A (PavA) and B (PavB) proteins.<sup>170,171</sup> In murine infection models, PavA-deficient pneumococci had impaired adherence to murine epithelium and endothelial cells and were unable to sustain long-term nasopharyngeal colonization.<sup>172,173</sup> Furthermore, although pneumococci lacking PavA showed similar growth to WT pneumococci in a sepsis model, PavA mutants were rapidly cleared from the central nervous system (CNS) after intracranial infections.<sup>172</sup> Possibly, PavA not only serves to directly bind fibronectin but also plays a role in the effective adherence and virulence mediated by other, so far unknown determinants.<sup>173</sup>

## **Bloodstream survival**

### **Complement system**

Once in the bloodstream, pneumococci are confronted with additional host defense mechanisms. Complement represents the first step of innate immunity against bacteremia. The classical complement pathway plays a dominant role in pneumococcal clearance, although the classical and alternative complement pathways are also activated by streptococcal species.<sup>174,175</sup> Pneumococci have developed two ways to minimize complement-mediated opsonization and phagocytosis. First, pneumococci undergo a second phase variation and become encapsulated. The polysaccharide capsule serves as a nonspecific barrier, significantly reducing complement deposition on the bacterial surface and limiting subsequent interaction with phagocytes.<sup>152,176</sup> In murine studies, systemically administered unencapsulated pneumococci were shown to be avirulent.<sup>134</sup>

Second, pneumococcal surface proteins PspA, PspC, and pneumolysin target specific complement components, thereby reducing complement-mediated bacterial clearance. PspA, which is expressed ubiquitously among pneumococci, inhibits C1q and subsequent C3b deposition.<sup>174</sup> PspC binds human factor H, thereby blocking the formation of C3 convertase (C3bBb), leading to lower C3b production and limiting opsonophagocytosis.<sup>177,178</sup> Pneumococci can also attach to erythrocytes through a process called immune adherence, which is dependent on the binding of complement components C3b, C4b, C1q, and MBL to both the pneumococcus and erythrocyte receptor CRI.<sup>177,179,180</sup> Immune complexes containing pneumococci, bound by complement to erythrocytes, are then transferred to macrophages, after which the erythrocytes are returned to the circulation.<sup>181</sup> Recent *in vitro* studies showed that PspA and PspC work synergistically to limit complement-mediated adherence and transfer to phagocytes.<sup>177</sup>

Pneumolysin, released during pneumococcal autolysis, readily binds the Fc portion of IgG, thereby potentially activating the classical complement pathway, increasing bacterial virulence by independently depleting complement factors away from the bacterium, and limiting opsonophagocytosis.<sup>182</sup> Murine bacteremia studies showed that pneumolysin-deficient pneumococci are either cleared from the bloodstream or allowed to develop into chronic bacteremia.<sup>183</sup> Furthermore, serum complement depletion may be particularly important in circumstances of overall limited complement availability, such as liver cirrhosis, and may further increase pneumococcal virulence at sites of limited complement presence, such as the nasopharynx.<sup>184,185</sup>

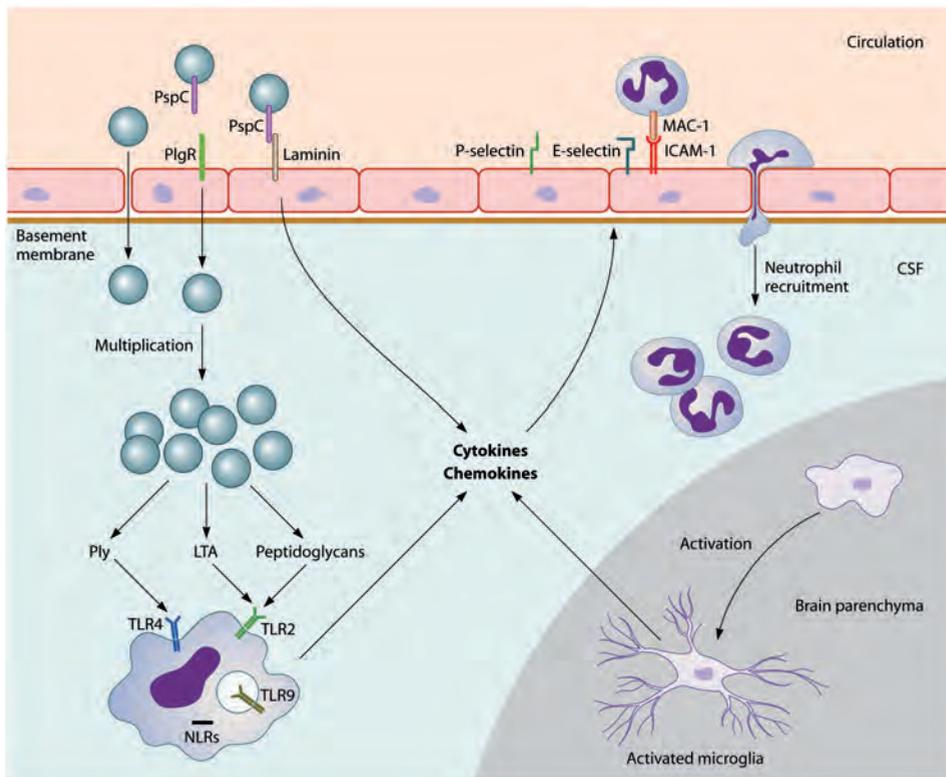
Lastly, the acute-phase CRP binds phosphorylcholine (Chop) on the PCW and subsequently interacts with C1q, leading to the activation of the classical complement pathway.<sup>186-189</sup> In mice, CRP is not an acute-phase protein, and treatment with human CRP reduced mortality following pneumococcal infection.<sup>190,191</sup> *In vitro* studies showed that CRP reduced pneumococcal binding to the epithelial cell PAF receptor.<sup>192</sup>

### Recognition by the host immune system

Pneumococci are recognized by APCs through the binding of pattern recognition receptors, which are specifically directed toward general motifs of molecules expressed by pathogens that are essential for pathogen survival. Pattern recognition receptors involved in sensing pneumococci include TLR2, TLR4, TLR9, and nucleotide oligomerization domain 1 (Nod1).<sup>193-204</sup> Upon activation of these receptors, APCs release various cytokines, which induce a cascade of inflammatory reactions, including the recruitment of neutrophils.<sup>48</sup> The most important cytokines released by phagocytic cells are tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin-1 (IL-1), and IL-6.<sup>205</sup> IL-1 $\beta$  and TNF- $\alpha$  act on local vascular endothelial cells, increasing vascular permeability and vasodilatation and upregulating adhesion molecules such as E-selectin, P-selectin, and vascular cell adhesion molecule 1 (VCAM-1) to enable the influx of neutrophils and other lymphocytes from the blood to the site of infection (Fig. 2).<sup>206,207</sup>

### Initiation of coagulation

Most patients with invasive pneumococcal disease show evidence of coagulation activation.<sup>208,209</sup> Inflammation-induced thrombin generation is not dependent on direct interaction of bacteria and the coagulation cascade but rather on the exposure of blood to tissue factor (TF).<sup>210</sup> TF is expressed primarily on cells outside the vasculature and is exposed to coagulation factors during vascular damage.<sup>211,212</sup> Low levels of circulating TF have been detected in healthy individuals, in whom the role of TF in thrombin generation remains uncertain.<sup>213-216</sup> The expression of TF in blood cells is limited to monocytes and can be elevated considerably during inflammation or sepsis.<sup>217</sup> The upregulation of TF is largely IL-6 dependent, as studies have shown abrogation of TF-dependent thrombin generation when IL-6 is blocked.<sup>218</sup>



**Figure 2.** *S. PNEUMONIAE* adheres to endothelial cells by using PspC, which binds laminin and pIgR, enabling transcytosis across the endothelium. Once in the CSF, pneumococci multiply freely and release bacterial products such as LTA and Ply, which are recognized by TLR2 and TLR4 on circulating APCs. The subsequent release of proinflammatory cytokines and chemokines from macrophages and microglial cells results in upregulation of endothelial cell P- and E-selectin and ICAM (which binds MAC-1 on leukocytes), leading to increased neutrophil recruitment into the CSF.

Upon exposure to blood, TF forms a complex with factor VII and catalyzes the conversion of factor X into factor Xa. Factor Xa allows prothrombin conversion to thrombin, although this reaction occurs to a significant extent only after thrombin-induced feedback activation

of factor VIII and factor V, nonenzymatic cofactors in the tenase and prothrombinase complexes, respectively.<sup>210,214</sup> The prothrombinase and tenase complexes convert prothrombin (factor II) into thrombin (factor IIa), which then leads to the conversion of fibrinogen to the clot-forming fibrin protein.<sup>219</sup> The activity of prothrombinase and tenase complexes is markedly enhanced by the presence of activated platelets, which become activated during inflammation but may also be activated directly by thrombin itself.<sup>220</sup>

Inflammation-mediated thrombin formation is regulated by three anticoagulant mechanisms: antithrombin (AT), the protein C system, and tissue factor pathway inhibitor (TFPI), all of which may be impaired during systemic infection.<sup>210</sup> Antithrombin inhibits thrombin and factor Xa, though during severe infection antithrombin levels are markedly lower due to impaired synthesis, degradation, and consumption during thrombin generation.<sup>221</sup> Circulating protein C, which upon conversion to activated protein C by the thrombin-thrombomodulin complex degrades the essential coagulation factors Va and VIIa, is hampered during severe inflammation by enzymatic degradation by neutrophil-derived elastase and by impaired synthesis as well as decreased activation by depressed levels of thrombomodulin.<sup>222,223</sup> Lastly, the importance of TFPI has been demonstrated in studies in healthy human volunteers injected with endotoxin, in whom administration of TFPI induced a marked inhibition of coagulation.<sup>224</sup> Animal studies showed that rabbits deficient in TFPI were more susceptible to severe disseminated intravascular coagulation (DIC), and primates infused with TFPI were able to survive exposure to otherwise lethal amounts of *Escherichia coli*.<sup>225</sup>

The degradation of fibrin clots is mediated by plasmin, the active form of plasminogen, which is activated by tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), both of which are stimulated by the inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ .<sup>226</sup> During severe infection, these cytokines subsequently induce plasminogen activator inhibitor type 1 (PAI-1), thereby limiting fibrinolysis and resulting in a net procoagulant state.<sup>226</sup> Higher levels of PAI-1 in patients with meningococcal septicemia or disseminated intravascular coagulation have been shown to be associated with poor outcomes and mortality.<sup>227,228</sup>

At relatively high concentrations, thrombin forms a complex with thrombomodulin and activates thrombin-activatable fibrinolysis inhibitor (TAFI; also known as plasma carboxypeptidase B, carboxypeptidase U, and carboxypeptidase R).<sup>229,230</sup> Activated TAFI inhibits fibrinolysis by limiting plasmin formation through the inhibition of plasminogen and tPA incorporation into fibrin clots.<sup>231</sup> Furthermore, TAFI is able to inhibit several proinflammatory substrates, such as bradykinin and complement components C3 and C5a.<sup>232</sup> The importance of TAFI and C5a was first demonstrated in a mouse model in which TAFI knockout mice showed a higher mortality when challenged with sublethal doses of lipopolysaccharide (LPS) and cobra venom factor.<sup>233</sup>

## Central nervous system invasion

### Intracellular translocation across the blood-brain barrier

Cerebral vascular endothelial cells show marked differences from their systemic counterparts. They exhibit very tight junctions, low rates of pinocytosis, and relatively large numbers of mitochondria.<sup>234</sup> In human brain microvascular endothelial cell cultures, the pneumococcus was able to adhere to the vascular endothelial PAF receptor, allowing transmigration through the endothelial cell to the basolateral site.<sup>235</sup> This mechanism of transcytosis is similar to that seen at the pulmonary epithelium (see Invasive Disease) and is mediated by binding of pneumococcal phosphorylcholine to the PAF receptor.<sup>106,125</sup> Pneumococci in the transparent phase are more efficient at invading the brain endothelial cell layer than opaque variants, which are dependent on the expression of phosphorylcholine.<sup>235</sup> Concordantly, PAF receptor-deficient mice showed less translocation of pneumococci across the blood-brain barrier and, therefore, a decreased incidence of pneumococcal meningitis after intravenous challenge.<sup>153</sup> Many of these studies have been performed with brain vascular endothelial cells. However, another important site of entry might be the choroid plexus epithelium, as shown for *Streptococcus suis*, which induces epithelial cell death and blood-brain barrier disruption in porcine choroid plexus epithelium but may also translocate intracellularly across the plexus epithelium.<sup>236,237</sup>

Nasopharyngeal colonization models demonstrated binding of pneumococcal PspC to pIgR on local epithelial cells, facilitating pneumococcal invasion.<sup>154</sup> However, in a cell line of human brain microvascular endothelial cells, the pIgR was not expressed.<sup>154</sup> *In vitro* and animal experiments showed that pneumococcal PspC may bind the laminin receptor on brain microvascular endothelial cells.<sup>238</sup> This receptor, by which endothelial cells are bound to the major component of basement membranes, laminin, was also shown to be a ligand for neurotropic viruses and prions.<sup>238-240</sup> Laminin appears to be involved in binding of bacteria that may cause meningitis, such as *S. pneumoniae*, *N. meningitidis*, and *H. influenzae*, to brain microvascular endothelial cells.<sup>238</sup> Pneumococcal PspC binds to laminin, and in a mouse model of pneumococcal sepsis, a pneumococcal PspC mutant caused a decreased frequency of pneumococcal meningitis.<sup>238</sup> These results indicate that the interaction between laminin and pneumococcal PspC plays a role in intracellular translocation of pneumococci across the blood-brain barrier.

### Intercellular Translocation across the Blood-Brain Barrier

Pneumococci may translocate into the CSF intercellularly, by disruption of the interepithelial tight junctions. In an animal model of pneumococcal meningitis, tight junctions between brain microvascular endothelial cells became disrupted in the course of the disease.<sup>234</sup> This may be due to damage caused by the pneumococcus or by factors of the host immune response.<sup>241-243</sup> Analogous to the nasopharyngeal setting, pneumolysin was capable of disrupting an endothelial cell layer in an *in vitro* endothelial cell culture, which may enhance blood-brain barrier disruption *in vivo*.<sup>243</sup>

After crossing the dense vascular endothelial cell lining, pneumococci have several methods of disrupting and invading the basement membrane. The first involves binding of plasminogen to the bacterial surface, which may subsequently be activated by tPA.<sup>244</sup> In patients with bacterial meningitis, levels of uPA correlated with breakdown of the blood-brain barrier and pleocytosis.<sup>245</sup> *In vitro* models showed that pneumococcus-mediated activation of plasminogen resulted in damage of extracellular matrix components and the basement membrane, although conversely, an *in vivo* mouse model failed to demonstrate an effect of tPA or uPA receptor on pneumococcal transmigration across the blood-brain barrier.<sup>244</sup> Finally, pneumococci may bind fibronectin, vitronectin, and collagen in the extracellular matrix, which may enhance blood-brain barrier disruption.<sup>246-248</sup>

## Central nervous system immune response

### Immune activation

During multiplication, pneumococci concurrently undergo autolysis, which eventually leads to a stationary phase where multiplication and autolysis rates are similar.<sup>249</sup> The released bacterial products are highly immunogenic and may lead to an increased inflammatory response in the host.<sup>250</sup> Bactericidal antibiotics causing bacterial lysis may also induce a similar effect and lead to a temporarily increased host inflammatory response and increased disease severity.<sup>251-253</sup>

A variety of pneumococcal compounds are proinflammatory. The pathophysiological aspects of the different compounds may be reproduced by intracisternal inoculation of heat-killed unencapsulated pneumococci, purified PCW, cell wall lipoteichoic acid, or cell wall peptidoglycan.<sup>254</sup> Heat-killed encapsulated pneumococci or purified pneumococcal capsular polysaccharides inoculated intracisternally into rabbits did not cause meningitis, indicating that the pneumococcal capsule is not immunogenic in the CSF.<sup>254</sup> Inoculation with knockout pneumococcal strains is another way to study the immunogenicity of pneumococcal compounds. In a murine model of pneumococcal meningitis, intracisternal inoculation with pneumolysin-deficient pneumococci resulted in lower bacterial loads, better clinical scores, and longer survival of the host.<sup>255</sup> However, histological inflammatory changes in this study were similar to those induced by wild-type pneumococci.<sup>255</sup>

### Anatomical localization of blood-brain barrier invasion by leukocytes

Neutrophils are thought to cross the blood-brain barrier mainly at the venous side of the penetrating cerebral blood vessels.<sup>256</sup> Here they migrate to the perivascular space, which is continuous with the subarachnoid space. However, some neutrophils penetrate the brain parenchyma. Neutrophilic infiltrates in the brain have been seen primarily in spaces adjacent to CSF, such as the corpus callosum, periventricular space, and the meninges.<sup>257</sup> Neutrophils mediate bacterial killing by phagocytosis of opsonized bacteria.<sup>48</sup> Phagocytosis is initiated by recognition and binding of bacteria by a neutrophil

and is facilitated by opsonization of the bacteria by complement and antibody. Following binding, the neutrophil engulfs the bacteria, after which the cell membrane closes around the pathogens and is cut off, forming a free membrane-covered entity within the cell called an endosome.<sup>48</sup> In the activated neutrophil, the endosome containing the pathogens is fused with a lysosome present in the cell, which contains several bactericidal mediators, including nitric and oxygen species, but also activated lysozymes, and the bacteria are killed. In addition to intracellular killing, neutrophils also secrete nitric and oxygen species, establishing a bactericidal milieu around the cell.<sup>48</sup> Adversely, these nitric and oxygen species may damage the surrounding tissue when they are present in large amounts and may be responsible, at least in part, for the neuronal damage seen in pneumococcal meningitis. This topic is discussed further in Neuronal Damage and Histopathology.

**Table 1:** Effects of pattern recognition receptor knockout or deficiency.

<b>Model/setting*</b>	<b>Outcome</b>	<b>Reference</b>
TLR2 KO mice	Higher cerebellar and blood bacterial titers, increased disease severity, no difference in cytokine response	264
	Significantly increased disease severity, higher CSF bacterial loads, and earlier death	263
CD14 KO mice	Significantly increased disease severity, higher CSF bacterial loads, and earlier death	263
TLR2/CD14 double-KO mice	Significantly increased disease severity, higher CSF bacterial loads, and earlier death	263
TLR4 KO mice	No difference from WT mice	265
TLR2/TLR4 double-KO mice	Decreased inflammatory response and increased disease severity in TLR2 and TLR4 double mutants	265
TLR2/TLR4/TLR9 triple-KO mice	No differences in immune response, bacterial load, or survival compared with TLR2/TLR4 double-knockout mice	265
Nod2-deficient microglial and astroglial cell line	Reduced levels of TNF- $\alpha$ and IL-6 production	266
Nod2 KO mice	Decreased MIP-1 $\alpha$ and TNF- $\alpha$ production and decreased cerebral demyelination and gliosis	266
SIGN-R1 on primary mouse and rat microglial cells	Involved in the uptake of pneumococcal capsular polysaccharides into the cell	289
Caspase-1 KO mice	Less severe inflammation and improved survival in a mouse model of pneumococcal meningitis	282
IRAK-4 deficiency in children	Increased susceptibility to invasive pneumococcal infections, including meningitis	293
MyD88 deficiency in children	Increased susceptibility to invasive pneumococcal infections, including meningitis	294
NEMO deficiency in patients	Increased susceptibility to invasive pneumococcal infections, including meningitis	297, 298
MyD88 KO mice	Increased mortality due to pneumococcal sepsis and meningitis, accompanied by decreased symptoms of infection and inflammatory parameters	269, 299

\*KO, knockout.

### Pattern recognition receptors

Immune activation in the cerebrospinal fluid is initiated by the recognition of different bacterial pathogen-associated molecular patterns (PAMPs) by APCs (Table 1).<sup>258,259</sup> These APCs are present at low levels in the CSF, or are situated in the meninges, choroid plexus, perivascular space, or brain parenchyma as astrocytes and microglial cells.<sup>260-262</sup> Major pattern recognition receptors involved in initial sensing of pneumococci in the CNS are TLR2, TLR4, TLR9, and Nod-like receptors (NLRs) (Fig. 3).<sup>202,263-266</sup>

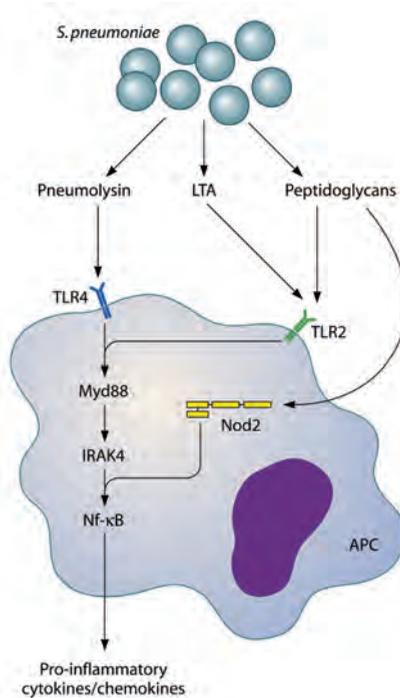
TLR2 recognizes PCW LTA.<sup>196,267</sup> TLR2 signaling is enhanced by the TLR2 coreceptor, CD14, and by LPS binding protein (LBP).<sup>196,198</sup> In a model of pneumococcal meningitis, TLR2-deficient mice showed increased disease severity with increased blood-brain barrier disruption and intracranial complications and increased bacterial loads.<sup>263,264</sup> Cytokine production was similar in TLR2-deficient and wild-type mice with pneumococcal meningitis, except for that of TNF- $\alpha$ , which was significantly higher in TLR2-deficient mice.<sup>263,268</sup> Since the phenotype of TLR2-deficient mice with pneumococcal meningitis was not as severe as that seen with mice lacking MyD88, an important general adaptor molecule for TLR signaling, it was proposed that other TLRs besides TLR2 may play a role in sensing pneumococci in the CNS.<sup>264,269</sup> TLR4 recognizes pneumococcal pneumolysin.<sup>201</sup>

TLR4-deficient mice did not differ significantly from wild-type mice in their host immune response, cerebrovascular changes, or outcome during pneumococcal meningitis.<sup>265</sup> However, in mice deficient in both TLR2 and TLR4, a marked reduction in inflammatory mediators, increased bacterial replication in the CNS, and reduced survival were seen compared to those for wild-type mice or mice with a single TLR deficiency.<sup>265</sup> Thus, in meningitis, both TLR2 and TLR4 are important receptors in detecting the pneumococcus and initiating a robust inflammatory response to the pathogen, and one receptor may compensate for the absence of the other.<sup>265</sup>

TLR9 is an intracellular pattern recognition receptor and is activated by CpG repeats in bacterial DNA.<sup>270</sup> *In vitro*, *S. pneumoniae* was able to activate alveolar and peripheral macrophages through TLR9 and induced IL-8 production in TLR9-transfected human embryonic kidney cells.<sup>195,202</sup> *In vivo*, TLR9-deficient mice showed reduced resistance to *S. pneumoniae* after intranasal challenge.<sup>202</sup> However, in a model of pneumococcal meningitis, triple mutant TLR2/TLR4/TLR9-deficient mice did not show significant differences in immune response, bacterial load, or survival compared with TLR2/TLR4-deficient mice.<sup>265</sup> Therefore, TLR9 appears to play a minor role in pneumococcal meningitis, although this was assessed only in TLR triple-knockout mice.

NLRs are a second group of intracellular pattern recognition receptors involved in detecting pneumococci.<sup>203</sup> NLRs belong to a family of receptors which, upon activation, induce activation of NF- $\kappa$ B or mitogen-activated protein kinase (MAPK) pathways and inflammatory caspases.<sup>203</sup> In human embryonic kidney 293 cells, Nod2 was activated by internalized pneumococci through sensing of *meso*-diaminopimelic acid (*meso*-DAP) motifs of the bacterial peptidoglycan.<sup>203,271</sup> *In vitro* experiments showed that microglial and astroglial cells are activated by *S. pneumoniae* through Nod2.<sup>266</sup> Murine microglial and

astroglial cells deficient in Nod2 showed reduced levels of TNF- $\alpha$  and IL-6 production.<sup>266</sup> With *in vivo* experiments using a pneumococcal meningitis model, Nod2 activation of primary murine glial cells induced macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) and TNF- $\alpha$  production and enhanced cerebral demyelination and gliosis.<sup>266</sup> Thus, activation of Nod2 appears to be one of the contributing factors leading to cerebral damage in bacterial meningitis.



**Figure 3:** Host pattern recognition receptors involved in sensing *S. pneumoniae*. TLR2 is activated by pneumococcal cell wall peptidoglycan and LTA. Nod2 is activated by cell wall peptidoglycans and TLR4, which in turn is activated by Ply. TLR2 and -4 activate the transcription factor NF- $\kappa$ B via MyD88 and IRAK-4. Nod2 also activates NF- $\kappa$ B, inducing transcription of several proinflammatory cytokines.

Another group of NLRs are the inflammasomes, which include a complex of various pattern recognition receptors sharing the caspase adaptor apoptosis-associated speck-like protein (ASC) and leading to caspase-1 activation when triggered.<sup>272</sup> Cleavage and activation of caspase-1 lead to cleavage of different procytokines into their active forms, including IL-1 $\beta$  and IL-18.<sup>273-275</sup> In addition, inflammasome activation may lead to a specific form of controlled cell death, different from apoptosis, called pyroptosis.<sup>276</sup> Inflammasomes are intracellular pattern recognition receptors and can be activated by several endogenous and exogenous ligands, including bacteria, bacterial DNA, bacterial toxins, endogenous reactive oxygen species (ROS) produced by macrophages in response to infection, and uric acid released through cell injury during inflammation.<sup>277-281</sup> Little is known about the role of inflammasomes in bacterial meningitis. In patients suffering from

bacterial meningitis, cerebrospinal fluid levels of caspase-1 were increased.<sup>282</sup> In children with bacterial meningitis, as well as a rat model of pneumococcal meningitis, increased IL-1 $\beta$  levels were measured in the CSF.<sup>283,284</sup> Koedel et al. showed that mice lacking caspase-1 displayed less severe inflammation and improved survival in a pneumococcal meningitis mouse model.<sup>282</sup> Similar results were found in a pneumococcal meningitis model with IL-18 knockout mice, indicating a role for inflammasome activation in the pathophysiology of pneumococcal meningitis.<sup>285</sup>

A fourth group of pathogen recognition receptors involved in sensing *S. pneumoniae* are the C-type lectins, which are highly expressed on splenic dendritic cells and also on peritoneal macrophages.<sup>286</sup> A member of this group, SIGN-R1, was shown to facilitate phagocytosis by recognition of the pneumococcal capsular polysaccharide.<sup>286,287</sup> Mice lacking functional SIGN-R1 fail to effectively phagocytose *S. pneumoniae*, leading to an inability to clear the infection and resulting in increased inflammatory parameters and reduced survival in both a model of pneumococcal peritoneal sepsis and one of intranasally induced pneumonia.<sup>286,288</sup> Furthermore, SIGN-R1 plays a role in the activation of the classical complement pathway by binding C1q.<sup>58</sup> Park et al. showed the presence of SIGN-R1 on microglial cells in mouse and rat brains, which was functionally active in taking up pneumococcal capsular polysaccharides into the cell.<sup>289</sup> Therefore, SIGN-R1 may be an important pathogen recognition receptor in the brain during pneumococcal meningitis.

### Downstream signaling molecules

Upon stimulation of one of the above pattern recognition receptors, an intracellular cascade is activated and leads to the production of inflammatory molecules, usually cytokines or chemokines, which modulate the immune response by activating or attracting specialized immune cells. Deficiencies and polymorphisms in the pathogen recognition receptor downstream signaling cascade in humans have been associated with invasive pneumococcal disease, including meningitis.

The most extensively characterized TLR downstream signaling protein in pneumococcal invasive disease is IRAK-4 (Fig. 3).<sup>290</sup> This adaptor protein is one of the links in TLR- and IL-1 receptor (IL-1R)-induced activation of MyD88 and NF- $\kappa$ B, which ultimately results in cytokine production.<sup>291,292</sup> Specifically, children with IRAK-4 deficiency are susceptible to (recurrent) invasive pneumococcal infections, which are associated with high mortality.<sup>293</sup> In a group of pediatric patients with normally expressed IRAK-4 but with recurrent invasive pneumococcal disease, deficiencies in the common adaptor molecule of TLR and IL-1R pathways, MyD88, were found.<sup>294</sup> Deficiencies in IRAK-4 and MyD88 give indistinguishable phenotypes. Both patient groups are unresponsive to all TLR1, -2, -5, -6, -7, and -8 agonists, TLR9 agonists, and IL-1R agonists.<sup>294-296</sup> In IRAK-4- or MyD88-deficient patients, the TLR3 signaling pathway is not affected, and the TLR4 pathway is affected only partially. Both TLR3 and -4 can still signal through the MyD88-independent TRIF pathway, leading to cytokine production.<sup>294</sup>

Stimulation of whole blood of IRAK-4- or MyD88-deficient patients with several different TLR agonists showed impaired production of IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, monocyte chemoattractant protein 1 (MCP-1), MIP-1 $\alpha$ , and MIP-1 $\beta$ .<sup>294</sup> Stimulation with a TLR3 or TLR4 agonist showed impaired production of IL-6, IL-10, and IL-12, as well as that of IL-8 in the case of TLR3 stimulation and IL-1 $\beta$  in the case of TLR4 stimulation.<sup>294</sup> Among patients with an IRAK-4 or MyD88 deficiency, 68% suffer from invasive pneumococcal disease, and *S. pneumoniae* is responsible for 53% of all episodes of infectious episodes in these patients.<sup>290</sup> Invasive bacterial disease in these patients consists of meningitis in 41% of IRAK-4-deficient patients and 52% of MyD88-deficient patients.<sup>290</sup> IRAK-4 and MyD88 appear to be specifically important at a young age, as no fatal disease has been reported after the age of 8 years, with no invasive infections after the age of 14 years.<sup>290</sup> Two patients have been described as having a homozygous mutation in the gene encoding NEMO, an adaptor molecule of the MyD88-dependent TLR, IL-1R, and TNF receptor (TNF-R) signaling pathways, and this mutation is associated with invasive pneumococcal disease.<sup>297,298</sup>

In mice, MyD88 deficiency resulted in increased susceptibility to systemic infection after colonization and increased mortality due to pneumococcal sepsis and meningitis.<sup>269,299</sup> Pneumococcal infection in MyD88<sup>-/-</sup> mice was accompanied by decreased symptoms of infection and inflammatory parameters, similar to the phenotype seen in patients lacking functional MyD88 or IRAK-M.<sup>269,290,297</sup> Deficiencies in the TLR and IL-1R signaling pathways have been associated with recurrent pneumococcal disease, illustrating the importance of these pathways in controlling pneumococcal infection.<sup>69</sup>

### Proinflammatory cytokines

The early response cytokines IL-1, TNF- $\alpha$ , and IL-6 are produced after pneumococcal recognition.<sup>300,301</sup> Several cells have been found to be capable of sensing pneumococci and produce proinflammatory cytokines: perivascular and meningeal macrophages, vascular endothelial cells, astrocytes, and microglial cells.<sup>241,302-306</sup> These early-phase cytokines induce upregulation of several adhesion factors on the vascular endothelium, mediating leukocyte influx (see above).<sup>206,207</sup> The majority of leukocytes recruited to the CSF are polymorphonuclear neutrophils, and influx occurs largely in the first 6 h of infection.<sup>303</sup>

TNF- $\alpha$  is an important early proinflammatory response cytokine. Patients with bacterial meningitis have increased CSF TNF- $\alpha$  levels early in the course of disease.<sup>242,307-310</sup> Intrathecal levels of TNF- $\alpha$  correlated with severity of blood-brain barrier disruption, disease severity, and neurologic sequelae in a study including 48 patients with bacterial meningitis.<sup>242</sup> In this study, TNF- $\alpha$  levels decreased within 24 h after the onset of antibiotic treatment.<sup>242</sup> In animal models of pneumococcal meningitis, TNF- $\alpha$  was produced mainly in the first 6 to 24 h of the immune response.<sup>311,312</sup> One hour after intrathecal injection of recombinant TNF- $\alpha$ , CSF leukocyte recruitment was observed in a rabbit model.<sup>313</sup> Intrathecal administration of anti-TNF- $\alpha$  antibody together with *S. pneumoniae* reduced CSF leukocytosis, protein content, and brain edema in these experiments.<sup>313</sup> TNF- $\alpha$

administered intravenously also mediated blood-brain barrier opening, facilitating bacterial traversal into the CSF.<sup>314</sup> However, TNF- $\alpha$  production is also essential for defense, as TNF- $\alpha$ -deficient mice showed decreased survival in a pneumococcal meningitis model.<sup>315</sup> Thus, TNF- $\alpha$  has been shown to be a marker of the acute inflammatory response and is associated with inflammation-related complications of bacterial meningitis but is also essential for an adequate host response to the infection.

IL-1 $\beta$  is a proinflammatory cytokine produced by, e.g., perivascular and meningeal macrophages.<sup>303</sup> CSF IL-1 $\beta$  levels are increased in the first 18 h of infection.<sup>316</sup> Pro-IL-1 $\beta$  is cleaved into its active form by caspase-1, which is regulated by a group of different receptors called the inflammasome.<sup>275</sup> Reported data on the role of IL-1 $\beta$  in bacterial meningitis are somewhat contradictory. Levels of IL-1 $\beta$  were not associated with the degree of blood-brain barrier disruption in patients with bacterial meningitis.<sup>242</sup> However, a pneumococcal model using caspase-1 knockout mice showed decreased levels of IL-1 $\beta$  and decreased intracranial pressure (ICP), leukocyte recruitment, and brain edema compared to those in WT mice.<sup>282</sup> IL-1 $\beta$  administered intrathecally did not lead to CSF pleocytosis or brain edema in a rabbit model of pneumococcal meningitis.<sup>313</sup> However, antibodies against IL-1 $\beta$  decreased leukocyte influx induced by TNF- $\alpha$ .<sup>313</sup> Mice deficient in the receptor for IL-1 $\alpha$  and IL-1 $\beta$  (IL-1R) showed impaired survival and decreased cytokine responses without alterations in CSF pleocytosis.<sup>317</sup> Thus, although IL-1 $\beta$  did not influence CSF pleocytosis in pneumococcal meningitis, other caspase-1-cleaved cytokines may be responsible for the reduced pleocytosis observed in caspase-1 knockout mice.

IL-6 is a proinflammatory as well as anti-inflammatory cytokine and has been shown to be upregulated in the acute phase of many infection models.<sup>318</sup> In a mouse pneumococcal meningitis model, IL-6 knockout mice displayed increased CSF pleocytosis but decreased cerebral edema, blood-brain barrier disruption, and intracranial pressure.<sup>319</sup> This was also described for a model of pneumococcal pneumonia where IL-6 was shown to downregulate multiple proinflammatory as well as anti-inflammatory cytokines.<sup>320</sup> Thus, in pneumococcal meningitis, IL-6 attenuates CSF leukocyte recruitment but does not inhibit complications related to fluid shift.

Gamma interferon (IFN- $\gamma$ ) is one of the major cytokines of the T-helper 1 (Th1) pathway. IFN- $\gamma$  was increased in the CSF of patients with pneumococcal meningitis.<sup>321,322</sup> IFN- $\gamma$  was also expressed in brain tissue of rats with pneumococcal meningitis.<sup>323</sup> The exact role of IFN- $\gamma$  in pneumococcal meningitis remains unclear. IL-12p70, an important stimulus for IFN- $\gamma$  production, could be detected in patients with pneumococcal meningitis and in animal models of pneumococcal meningitis.<sup>322</sup> Macrophage inflammatory factor (MIF) was found to be increased in the CSF of patients with pneumococcal meningitis and has also been associated with disease severity, suggesting a role for MIF in the pathophysiology of pneumococcal meningitis.<sup>324,325</sup>

### Anti-inflammatory cytokines

Anti-inflammatory cytokines include IL-10 and TGF- $\beta$ .<sup>326-329</sup> IL-6 may act partially as an anti-inflammatory cytokine and has been discussed earlier.<sup>320</sup> IL-10 is an anti-inflammatory cytokine with multiple effects, including downregulation of proinflammatory cytokines and costimulatory molecules on macrophages and impairment of neutrophil phagocytosis and killing.<sup>326,329,330</sup> IL-10 has been shown to downregulate TNF- $\alpha$ , IL-6, and keratinocyte-derived chemokine (KC), thereby reducing CSF pleocytosis in pneumococcal meningitis.<sup>331</sup> Nonetheless, in experimental pneumococcal meningitis, IL-10 knockout mice did not have altered bacterial loads or survival.<sup>331</sup> This anti-inflammatory cytokine has been described as an important repressor of sepsis-associated neuronal damage. Its pathophysiology is unclear, but it appears that inflammatory mediators as well as bacterial components cross the blood-brain barrier and induce a local inflammatory response.<sup>332-334</sup> In mice overexpressing IL-10, the development of sepsis-associated neuronal damage as a result of pneumococcal sepsis has been shown to be decreased.<sup>332</sup> In line with this, Koedel et al. showed that intravenously administered recombinant IL-10, as opposed to intracisternally administered IL-10, reduced the levels of CSF proinflammatory cytokines, CSF pleocytosis, cerebral edema, and intracranial pressure in a rat model of pneumococcal meningitis.<sup>335</sup> Interestingly, intracisternally administered IL-10 had the opposite effect, as it increased CSF pleocytosis in rats with pneumococcal meningitis and induced an inflammatory response in uninfected rats.<sup>335</sup> Thus, systemic IL-10 reduces cerebral inflammation and secondary complications in pneumococcal meningitis.

TGF- $\beta$  is an anti-inflammatory cytokine with multiple functions, including differentiation and maintenance of regulatory T cells (Tregs), differentiation of Th17 T cells, and inhibition of Th1 and Th2 T-cell maturation and differentiation, but TGF- $\beta$  also suppresses macrophage activation and production of several proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, and TNF, by microglial cells.<sup>327,328,336</sup> Activated Tregs produce TGF- $\beta$  in an autocrine fashion and are thought to modulate the immune response in such a way that the host's tissues are minimally damaged while the invading pathogen is effectively eliminated, by downregulating the acute inflammatory response. In a mouse model of pneumococcal meningitis, TGF- $\beta$  was associated with cerebral vasculitis, a frequent complication in patients with meningitis.<sup>6,337</sup> Mice with leukocytes deficient in TGF- $\beta$  receptor II (TGF- $\beta$ RII) showed increased neutrophil influx into the subarachnoid space, which was accompanied by increased bacterial clearance and survival of the host.<sup>337</sup> In addition, TGF- $\beta$ RII knockout mice showed decreased blood-brain barrier disruption, intracranial pressure, and cerebral vasculitis.<sup>337</sup> However, when TGF- $\beta$ 2 or TGF- $\beta$ 1 was administered intraperitoneally in a rat model of sterile meningitis induced by a PCW lysate, cerebral edema, intracranial pressure, and cerebral blood flow (CBF) decreased.<sup>338</sup> Thus, leukocyte TGF- $\beta$ RII signaling has an unfavorable effect on the course of pneumococcal meningitis, although systemic TGF- $\beta$  production appears to decrease the complications of meningitis.

### Chemokines

Chemokines are a subgroup of cytokines with chemotactic activity recruiting effector immune cells to the site of infection.<sup>48</sup> Multiple chemokines have been reported to be upregulated in the CSF of patients with pneumococcal meningitis, including MIP-1delta (CCL15), NAP-2 (CXCL7), MIP, MCP-2 (CCL8), PARC (CCL18), MIP-3α (CCL20), ENA-78 (CXCL5), GRO-α (CXCL-1), IL-8 (CXCL-8), MCP-1 (CCL2), MIP-1α (CCL3), and MIP-1β (CCL4).<sup>310,312,339-342</sup> In animal models of pneumococcal meningitis, additional chemokines have been identified by protein arrays for brain tissue, including MIP-1γ (CCL9), MIP-2 (CXCL-2), lymphotactin (XCL-1), TCA-3 (CCL1), eotaxin (CCL11), MCP-5 (CCL12), eotaxin-2 (CCL24), TECK (CCL25), PF-4 (CXCL4), CRG-2 (CXCL10), SDF-1α (CXCL12), BLC (CXCL13), and CXCL16.<sup>343</sup> The role in pneumococcal meningitis of many of these chemokines has not been elucidated yet.

IL-8 is one of the well-characterized chemokines involved in pneumococcal meningitis. IL-8 was found to be chemotactic for neutrophils in the CSF of patients with bacterial meningitis.<sup>340</sup> Furthermore, CSF IL-8 levels increased as a result of blocking leukocyte recruitment in rabbits with pneumococcal meningitis, indicating local production of chemotactic cytokines.<sup>344</sup> In patients with bacterial meningitis, no correlation was found between the CSF white blood cell (WBC) count and IL-8.<sup>340</sup> Ostergaard et al. showed that not intracisternal but rather systemic IL-8 levels induced CSF pleocytosis in a rabbit model of pneumococcal meningitis.<sup>345</sup> Thus, IL-8 appears to regulate CSF pleocytosis from the systemic compartment, comparable with the proinflammatory cytokine TNF-α and the anti-inflammatory cytokines IL-10 and TGF-β.

The CCL chemokines MCP-1, MIP-1α, and MIP-2 were produced *in vitro* by astrocytes and microglial cells in response to PCW structures.<sup>305,346</sup> *In vitro*, antibodies against MCP-1, MIP-1α, and MIP-1β inhibited monocyte chemotactic properties of CSF from patients with pneumococcal meningitis.<sup>340</sup> Furthermore, intracisternal inoculation of recombinant MIP-1 or MIP-2 induced blood-brain barrier disruption, CSF leukocytosis, and cerebral edema in a rabbit model of pneumococcal meningitis; blocking MIP-1 or MIP-2 delayed these inflammatory alterations by 2 h.<sup>313</sup> Another experiment showed that blocking the receptor for MIP-1, i.e., CCR2, specifically reduced the influx of monocytes into the subarachnoid space in a mouse model of pneumococcal meningitis, while not changing bacterial clearing.<sup>347</sup> Thus, both MIP-1 and MIP-2 are produced by immune cells resident in the brain and attract monocytes and neutrophils from the bloodstream into the CSF in the acute stage of infection. The role of MCP-1 in pneumococcal meningitis has not been studied extensively.

Of the CXCL chemokines, ENA-78 was found to be upregulated in patients with bacterial meningitis and exhibited specifically neutrophil chemotactic properties together with IL-8.<sup>341</sup> GRO-α was also found at high levels in the CSF of patients with bacterial meningitis, as well as in a rat model of pneumococcal meningitis, but it did not exert any chemotactic activity.<sup>283,341</sup> In summary, multiple chemokines have been shown to be upregulated in pneumococcal meningitis. Most of them have a role in attracting leukocytes

to the CSF. However, the roles of many other chemokines have not been investigated extensively.

### Leukocyte migration adhesion molecules

In response to proinflammatory cytokines, selectins and integrins are upregulated on the blood endothelium and leukocytes are attracted from the bloodstream.<sup>256,348</sup> Cerebral perivascular and meningeal macrophages play a key role in attracting leukocytes across the blood-brain barrier into the CSF.<sup>302</sup> About 90% of the attracted leukocyte population consists of neutrophilic granulocytes, with the other 10% being predominantly monocytes.<sup>268,302,347</sup> Rats depleted of perivascular and meningeal macrophages by use of clodronate showed decreased leukocyte recruitment into the CSF despite increased expression of MIP-2, IL-6, and VCAM-1.<sup>302</sup> Furthermore, these depleted rats showed increased bacterial outgrowth in the CSF and poorer clinical scores than those for control rats with pneumococcal meningitis.<sup>302</sup> Thus, leukocyte attraction to the subarachnoid space seems to be crucial for efficacious clearing of *S. pneumoniae* from the subarachnoid space and dependent on perivascular and meningeal macrophage activation but appears to be mediated by cytokines other than IL-6 and MIP-2.

Other cytokines and chemokines attracting leukocytes to the subarachnoid space are TNF- $\alpha$ , IL-8 (systemic), MIP-2, and ENA-78 (see above).<sup>313,341</sup> Monocytes are also attracted from the bloodstream into the CSF but appear to play a minor role in the pathogenesis of pneumococcal meningitis.<sup>347</sup>

Leukocytes cross the blood-brain barrier by binding to selectins on the endothelium.<sup>256</sup> Binding to P- and E-selectin promotes leukocyte rolling across the endothelium.<sup>256</sup> Blocking L-selectin by fucoidin treatment reduced leukocytosis and disruption of the blood-brain barrier in rabbits challenged intrathecally with pneumococcal antigen.<sup>256</sup> Integrins are also upregulated on the vascular endothelium, facilitating binding of leukocytes and subsequent blood-brain barrier migration.<sup>284</sup> An important integrin involved in leukocyte recruitment in pneumococcal meningitis is ICAM-1, which is known to bind MAC-1 (CD11b/CD18) on the leukocyte surface.<sup>349,350</sup> Rabbits treated intravenously with antibodies against CD18 showed decreased CSF leukocytosis, blood-brain barrier permeability, and brain edema and improved survival after intracisternal challenge with PCW or *S. pneumoniae*.<sup>349,350</sup> Interestingly, antibodies directed against CD11b did not alter CSF leukocytosis in the same rabbit model of pneumococcal meningitis, which may implicate a role for CD11a/CD18 or CD11c/CD18. In line with this, CD11a/CD18-deficient mice showed increased rates of meningitis and otitis media following intraperitoneal infection with *S. pneumoniae*.<sup>350,351</sup>

The integrin ICAM-1 was shown to be expressed on brain vascular endothelial cells in response to PCW, through an autocrine loop involving TNF- $\alpha$ .<sup>241</sup> In a rat model of meningitis induced by PCW, antibodies against ICAM-1 reduced the increase in CBF, increase in ICP, brain edema, and CSF leukocyte counts observed in the first hours after

induction of meningitis.<sup>352</sup> In an infant mouse model of pneumococcal peritonitis, ICAM-1 deficiency did not reduce the incidence of meningitis, and histopathologically there was no difference in the severity of inflammation (469).<sup>353</sup> Thus, ICAM-1 is not solely responsible for leukocyte recruitment to the brain.

### Other chemoattractants

PAF is a protein produced by neutrophils and endothelial cells in response to inflammatory stimuli, and it facilitates adhesion of leukocytes to the vascular endothelium.<sup>354-356</sup> In rabbits, PAF administered intrathecally induced blood-brain barrier permeability and cerebral edema at doses much lower than those at which it induced leukocytosis.<sup>349</sup> Antibodies against CD18 blocked these effects.<sup>349</sup>

In response to pneumococci, endothelial cells and neutrophils are stimulated to produce reactive nitrogen species (RNS), such as NO, by endothelial nitric oxide synthetase (eNOS) and inducible nitric oxide synthetase (iNOS), respectively.<sup>241,357</sup> The cerebral vasculature appears to be the main location where ROS are active, and increased levels of ROS are associated with blood-brain barrier disruption.<sup>358,359</sup> In patients with meningitis, positive correlations were found between CSF derivatives of NO production and CSF leukocyte counts and protein concentrations in a group of 27 children with bacterial meningitis; however, only 2 of these children had confirmed pneumococcal meningitis.<sup>360</sup> Mice deficient for iNOS showed decreased blood-brain barrier disruption and decreased IL-1 $\beta$ , IL-6, TNF- $\alpha$ , MIP-1 $\alpha$ , and MIP-2 mRNA levels in the brain.<sup>357</sup> The opposite was true for eNOS-deficient mice, which showed more profound leukocyte infiltrates, increased cytokine levels, and decreased survival due to pneumococcal meningitis.<sup>361</sup> A third form of NOS, neuronal NOS (nNOS), appears to play a minor role in fluid balance-related complications of pneumococcal meningitis.<sup>362</sup> In addition to RNS, ROS such as O<sub>2</sub><sup>-</sup> are produced by the enzyme NADPH oxidase in neutrophils, macrophages, and endothelial cells in response to infection.<sup>363</sup> In mice deficient for the subunit of NADPH oxidase in nonphagocytic cells, such as endothelial cells (p47), detrimental effects on blood-brain barrier permeability, subarachnoid space inflammation, and bacterial outgrowth were found.<sup>363</sup> Mice deficient for the subunit of NADPH oxidase in phagocytic cells (gp91) did not show any inflammatory differences from WT mice in the course of pneumococcal meningitis.<sup>363</sup> Thus, RNS/ROS produced specifically by cerebral endothelial cells, as opposed to granulocytes and macrophages, contribute to the blood-brain barrier damage and associated complications observed during pneumococcal meningitis.

The fibrinolysis factor uPA is also implicated in leukocyte recruitment to the brain in pneumococcal meningitis. In a group of 12 patients with bacterial meningitis (67% of cases were caused by *S. pneumoniae*), CSF uPA levels were associated with leukocyte recruitment and blood-brain barrier disruption.<sup>245</sup> In this study, serum uPA levels correlated with unfavorable clinical outcomes for these patients with bacterial meningitis.<sup>245</sup> Mice deficient in uPA showed reduced CSF leukocytosis, although blood-brain barrier permeability, ICP, expression of chemokines, bacterial killing, and clinical

outcomes were not different from those for WT mice.<sup>364</sup> Interestingly, deficiency in tPA did not have any implications in a mouse pneumococcal meningitis model.<sup>364</sup>

### **The complement system**

A fourth chemoattractant factor for CSF leukocytes is the complement system. Low or undetectable CSF levels of C3, C4, and B were found in uninfected control subjects.<sup>365</sup> In response to infection, the liver produces an array of acute-phase proteins which includes several complement components.<sup>366,367</sup> Circulating monocytes, macrophages, and epithelial cells of the pulmonary and gastrointestinal tracts also produce substantial amounts of complement components.<sup>367-370</sup> In addition, brain resident macrophages and monocytes recruited to the CSF during meningitis may also locally produce complement components. C3 was also found to be produced by astrocytes and neurons in response to HIV or proinflammatory cytokines.<sup>371-373</sup> Cultured human brain pericytes from a patient with Alzheimer's disease have been shown to produce C1q, and activated astroglial cells can produce C1q, which has been associated with increased blood-brain barrier damage in a rat model of neurotoxicity.<sup>374,375</sup> Microglial cells have been shown to upregulate C1q, C3, C4, and C5a production in response to injury.<sup>376</sup> Thus, levels of complement components are increased in the peripheral blood but may also be produced locally in the brain during infection or inflammation.

During infection or inflammation, the immune response in the brain compartment may do more harm than good. Under normal circumstances, the brain expresses multiple inhibitory factors for complement activation. One of these, factor H, was found to be expressed constitutively in neurons, brain endothelial cells, microglial cells, and astrocytes in mice.<sup>377</sup> In a mouse model of antibody-mediated inflammation, expression of factor H in these cells was suppressed; when recombinant factor H was administered, complement opsonization, axonal injury, and leukocyte infiltration decreased.<sup>377</sup> Thus, in addition to monocytes and macrophages, brain resident cells may contribute to the production of complement factors leading to leukocyte influx during inflammation.

In a rabbit model of pneumococcal meningitis where cobra venom (known to consume complement factor 3) was administered systemically, treated mice showed decreased survival accompanied by increased bacterial outgrowth in the CSF.<sup>49</sup> CSF pleocytosis was similar between the treated and untreated groups, but neutrophils were severely impaired in phagocytosis and killing of bacteria.<sup>49</sup> In line with these results, mice deficient in C1q or C3 also showed increased bacterial outgrowth in the CSF in a pneumococcal meningitis model, which was accompanied by decreased survival.<sup>49,60</sup> C1q and C3 knockout mice displayed a tempered inflammatory response which was reflected by a decreased leukocyte count in the CSF, decreased brain cytokine and chemokine levels, and fewer meningitis-associated intracranial complications. However, survival was decreased in this model as a result of more fulminant sepsis accompanied by systemic complications.<sup>60</sup> Similar results were found in mice deficient for the C3b receptor (CR3), which is also an integrin involved in binding of leukocytes to the endothelium.<sup>378</sup>

CR3<sup>-/-</sup> mice showed increased bacterial outgrowth compared to WT mice, with decreased survival, as a result of decreased neutrophilic superoxidase production in the CR3<sup>-/-</sup> mice leading to ineffective bacterial killing, while CSF pleocytosis was not different between groups.<sup>378</sup> In rabbits, intracisternally administered C5a resulted in rapid CSF pleocytosis and increased CSF protein levels which peaked 1 h after injection.<sup>379</sup> Furthermore, the CSF of rabbits with pneumococcal meningitis lost its chemotactic activity to neutrophils after incubation with an antibody against C5a.<sup>380</sup>

## MMPs

Matrix metalloproteinases (MMPs) are Zn<sup>2+</sup>- and Ca<sup>2+</sup>-dependent endopeptidases capable of breaking down and remodeling extracellular matrix components such as fibronectin, laminin, proteoglycans, and type IV collagen.<sup>381,382</sup> MMPs are produced mainly by activated neutrophils and, to a lesser extent, also by macrophages, monocytes, and possibly TNF- $\alpha$ -stimulated endothelial cells.<sup>383,384</sup> Furthermore, constitutive MMP expression is found on microglial cells and astrocytes and may be modulated during neuroinflammation and meningitis.<sup>385-387</sup>

The action of MMPs was initially believed to be limited to the breakdown of ECM during leukocyte migration across the subendothelial layer.<sup>388</sup> However, experimental bacterial meningitis models revealed that inhibition of MMPs did not result in a reduction of CSF pleocytosis, although blood-brain barrier permeability disruption was attenuated.<sup>389</sup> More recent studies have revealed a wide range of MMP substrates, such as chemokines, growth factors, and adhesion molecules, as well as cytokines and cytokine receptors, allowing MMPs to influence the course of various inflammatory conditions.<sup>388,390</sup>

In patients with bacterial meningitis, CSF levels of MMP-8 and MMP-9 were elevated.<sup>309</sup> Moreover, higher levels of MMP-9 were detected in children with meningitis who developed hearing impairment or secondary epilepsy than in those who recovered without neurological deficit.<sup>309</sup>

To avoid unwanted proteolytic activity, the activity of all MMPs is tightly regulated by binding to inhibitory proteins called tissue inhibitors of metalloproteinases (TIMPs). MMP-9, for instance, forms complexes with and is inactivated by TIMP-1, both of which are upregulated during pneumococcal meningitis.<sup>389</sup> In a murine model of pneumococcal meningitis, the induction of TIMP-1 was delayed in relation to that of MMP-9, favoring increased collagen IV degradation and subsequent increased blood-brain barrier permeability.<sup>391</sup> Thus, treatment options including drugs specifically targeting MMPs are being investigated.<sup>392</sup> Interestingly, in a rat model of pneumococcal meningitis, adjuvant treatment of pneumococcal meningitis with dexamethasone resulted in lower MMP-9 mRNA expression, suggesting a possible mechanism of corticosteroids as an adjuvant treatment for bacterial meningitis.<sup>393</sup>

### Oxidative stress

One of the characterizing features of pneumococcal meningitis is the marked recruitment of leukocytes into the CSF.<sup>144,147</sup> The subsequent release of large amounts of RNS and ROS has been documented for patient populations as well as in animal models and plays a central role in the development of intracranial complications and brain damage.<sup>394-396</sup> In the past decade, ROS and RNS have been investigated extensively as potential targets for adjuvant treatment (reviewed by Klein et al.).<sup>397</sup>

During pneumococcal meningitis, RNS are produced by iNOS and eNOS.<sup>357</sup> NOS inhibition studies with experimental pneumococcal meningitis models have yielded contradictory results, at least in part due to a lack of drug specificity for single isoforms of NOS.<sup>398</sup> Moreover, iNOS and eNOS knockout studies show that the source of RNS is pivotal in determining its function during disease progression. Thus, NO derived from iNOS appears to contribute to blood-brain barrier disruption and to production of proinflammatory mediators (such as IL-1- $\beta$ , TNF- $\alpha$ , and MIP-2), whereas eNOS-derived NO plays a largely protective role.<sup>357,361</sup>

Reactive oxygen species, such as superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals, are produced by brain resident immune cells as part of the host response to invasive bacterial infections.<sup>399</sup> Pneumococci themselves are also an important source of  $H_2O_2$ , which not only is able to cause direct cytotoxic damage but also interacts with host NO to form the highly reactive species peroxynitrite ( $ONOO^-$ ).<sup>400-402</sup> When present in large quantities, ROS overwhelm the resident antioxidant mechanisms (such as superoxide dismutase and glutathione), leading to tissue exposure to oxidative stress.<sup>399</sup> Interventions aimed at scavenging ROS or enhancing antioxidant activity have generally resulted in reductions of intracranial complications such as elevated ICP, increased CBF, brain edema, and neuronal injury.<sup>397,398</sup>

Peroxynitrite, which is formed by the combination of superoxide radicals and NO, is a very reactive, short-lived molecule whose direct detection has proven difficult; the compound nitrotyrosine (which is formed by the reaction of  $NOO^-$  with tyrosine) is widely used as a marker.<sup>403</sup> In patients with bacterial meningitis, elevated CSF levels of nitrotyrosine were associated with an unfavorable outcome and with lower CSF concentrations of the antioxidant ascorbic acid, suggesting antioxidant depletion by the RNS.<sup>395</sup> Furthermore, in autopsy studies, nitrotyrosine was detected in the leptomeninges, subarachnoid granulocytes, and penetrating cortical and leptomeningeal vasculature.

Peroxynitrite can damage neurons and glial cells in two ways. First, it causes damage by means of lipid peroxidation and cell membrane destabilization, which occurs by peroxynitrite attack on lipid peroxidation and is consistently seen in brain homogenates of rats with pneumococcal meningitis.<sup>397,404</sup> Blocking of lipid peroxidation with aminosteroids limits neuronal damage.<sup>404</sup> Alternatively, peroxynitrite can cause DNA fragmentation and subsequent poly(ADP-ribose) polymerase (PARP) activation, which leads to cell energy depletion and cell death.<sup>398</sup> PARP knockout mice, as well as mice treated with a PARP

inhibitor, demonstrated lower levels of inflammation and a better clinical course during pneumococcal meningitis.<sup>398,404</sup>

Klein et al. have reviewed the mechanisms of oxidative damage (activation of cytokines and chemokines, neutrophil activation, lipid peroxidation, DNA and mitochondrial damage, tyrosine nitration, MMP activation/TIMP inactivation, K<sup>+</sup> channel activity alterations, and prostaglandin synthesis) as well as the resulting pathophysiologic alterations in pneumococcal meningitis.<sup>397</sup>

### Coagulation

The importance of coagulation and fibrinolytic dysregulation during pneumococcal meningitis is illustrated by the large number of cerebrovascular complications, which occur in up to one-third of patients.<sup>147</sup> Analysis of CSF in patients with bacterial meningitis revealed increased levels of both coagulation and fibrinolytic factors (Table 2).<sup>245,405</sup> More recently, PAI-1 was shown to be associated with the occurrence of brain infarctions, though no causal relationship was determined.<sup>406</sup> Furthermore, in a recent autopsy series of patients who died of pneumococcal meningitis, fibrin thrombi and cerebral infarctions were found in the absence of inflammatory vessel wall infiltrates, suggesting that disseminated cerebral intravascular coagulation might be an additional explanation for ischemic stroke in pneumococcal meningitis.<sup>407</sup> The precise mechanism of cerebral

Table 2: Coagulation studies.

Setting	Material	Factor	Change in concn	Ref	
Human studies	Thirty-eight patients with bacterial meningitis (GCS <sup>a</sup> of <9 vs GCS >9)	Serum	PLT/dPLT	↑	467
			PT <sub>r</sub>	↓	
			INR	↑	
			D-dimers	↑	
	Ninety-two patients with bacterial meningitis vs. controls and patients with viral meningitis.	CSF	sTF	↑	406
			TaT	↑	
			pT fragment F1+2	↑	
			t-PA	↑	
			PAI-1	↑	
			D-dimer	↑	
	Twelve patients with bacterial meningitis vs. 10 patients with GBS and 10 controls.	Serum/CSF	uPA	↑/↑	245
			uPAR	=/↑	
			PAI-1	=/↑	
			PA-dependent platelet activation	↑	
	Twelve patients with bacterial meningitis vs. 10 patients with GBS and 10 controls.	Serum/CSF	tPA	↑↑/↑	405
Murine studies	C57BL/6 tPA-/- uPAR-/-	Brain Frozen samples Section	tPA/uPA	↑/=	364
			PAI-1/2	↑/↑	
			uPAR	↑	
			MIP-2	↑	
			KC	↑	
			Albumin	↑	

<sup>a</sup>GCS, Glasgow coma score.

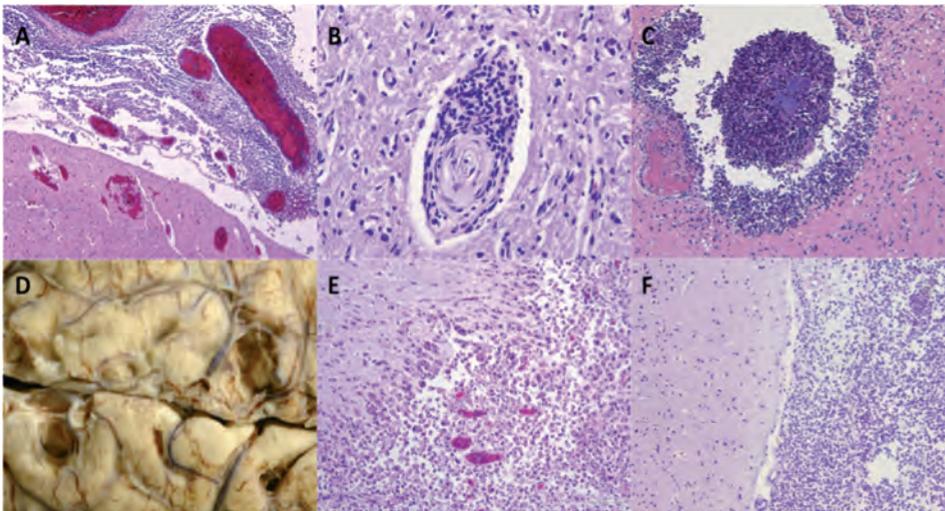
infarction remains unclear but may include mechanisms such as vascular endothelial swelling, local vascular inflammation, and cerebral intravascular coagulopathy.<sup>407,408</sup>

Microhemorrhages are also frequently observed in the leptomeninges, cortex, and white matter and are located mostly around congested small veins and capillaries.<sup>407</sup> It may be hypothesized that the massive clotting process results in local depletion of clotting factors, thereby inducing the local formation of microhemorrhages. In severe cases of disseminated cerebral intravascular coagulation, these microhemorrhages might potentially lead to clinically manifested intracerebral macrohemorrhages, which are rarely observed in patients with bacterial meningitis.

## Neuronal damage and histopathology

### Neuronal damage/histopathology

Human observational studies have repeatedly found long-term sequelae after pneumococcal meningitis, including sensomotor deficit, hearing loss, and cognitive impairment, which may occur in up to 30% of surviving patients.<sup>2,5,7,8,409,410</sup> Human histopathological data showed that the parenchymal damage was caused by increased ICP, cytotoxic and vasogenic edema, herniation, and local leukocyte infiltration or abscess formation, as well as by cortical necrosis and hippocampal neuronal loss.<sup>407,411</sup> Experimental animal models of pneumococcal meningitis have demonstrated large variations in histopathological features (Fig. 4 and Table 3), most likely due to different combinations of bacterial strains, infected animal species, methods of inoculation, and stages of infection.<sup>412,413</sup>



**Figure 4:** Neuronal damage and histopathology in humans with pneumococcal meningitis. The images show the histopathology of patients with bacterial meningitis, including parenchymal and meningeal hemorrhages (A), neutrophilic infiltration and arteritis obliterans (B), abscess formation and venous thrombosis (C), recent infarctions (D and E), and meningitis without cortical infiltration (F).

### Microglial activation

Microglial cells, a specific subset of cells related to monocytes and dendritic cells, form the initial line of defense of brain parenchyma against damage, injury, or infection and play a pivotal role in tissue repair, removal of dead debris, and recruitment of other immune cells to the site of infection.<sup>414-416</sup> Together with meningeal and perivascular macrophages, microglia express a wide palette of TLRs and become activated during bacterial infections.<sup>417</sup> Once activated, microglia are capable of producing large amounts of proinflammatory cytokines as well as reactive oxygen and reactive nitrogen intermediates, thus potentially playing both neuroprotective and neurotoxic roles.<sup>418-420</sup>

**Table 3:** Neuronal damage.

Process or type of damage	Outcome	References
Microglial activation	Microglial activation is induced by pneumococcus and mediated by TLRs 2, 4, and 9	421, 444, 419, 266
	Activin A may mediate microglial proliferation and activation	423, 424, 425
Neuronal apoptosis	The early phase of hippocampal apoptosis is caspase dependent and mediated by H <sub>2</sub> O <sub>2</sub> , pneumolysin, and AIF	436, 435, 401, 437, 185, 432, 433
	The late phase of hippocampal apoptosis is caspase independent and mediated by pneumococcal cell wall products, in a microglial TLR-2-dependent manner	400, 438, 439, 432
Sepsis-mediated neuronal damage	Neuronal damage may result in sepsis without concurrent bacterial growth in the CNS compartment	332, 445, 446, 447
Cochlear damage	Cochlear damage is correlated with CSF inflammation and is mediated by TLR and MyD88 signaling pathways	459, 451, 452, 460
	NO may contribute to cochlear damage; iNOS and eNOS are upregulated during pneumococcal meningitis, and damage can be attenuated using NO inhibitors/O <sub>2</sub> scavengers	462, 457, 410, 461

*In vitro* studies have demonstrated that microglial cells express TLRs 2, 4, and 9, which upon activation by specific TLR agonists induce proinflammatory cytokine and NO production, as well as increased phagocytic activity.<sup>306,421</sup> More specifically, the neurotoxic effects of CpG DNA, which is released into the subarachnoid space in large amounts by pneumococci during bacterial growth and following antibiotic treatment, were shown to be mediated largely via stimulation of TLR9 on microglial cells.<sup>419</sup> Furthermore, pneumolysin, which is a ligand for TLR4, was also found to be a potent activator of microglial cells, causing microglial cytotoxicity at high concentrations.<sup>421</sup>

Activin A, a member of the TGF- $\beta$  superfamily, is a neuroprotective cytokine which has been shown to be expressed constitutively in CSF and elevated in patients during bacterial meningitis.<sup>422,423</sup> Elevated activin A levels in CSF have also been detected in a rabbit meningitis model and were produced by cultured microglial cells following treatment with agonists of TLRs 2, 4, and 9.<sup>424,425</sup> Furthermore, *in vitro*-cultured murine microglial cells stimulated with LPS showed that cotreatment with activin A increased

microglial proliferation and negatively regulated production of NO, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ .<sup>423</sup>

Although no studies have been performed regarding the effects of dexamethasone on microglial activation during pneumococcal meningitis, *in vitro* studies using LPS-stimulated glial cells demonstrated that microglial activation after bacterial exposure is limited by corticosteroid treatment, providing a possible explanation for the observed beneficial effects of dexamethasone treatment *in vivo*.<sup>11,12,426,427</sup>

A recently published hypothesis suggested that microglia may respond differently to a stimulus preceded by another stimulus, a phenomenon called “priming”.<sup>390</sup> Aging itself may result in enhanced microglial activation following single or repeated stimulation.<sup>428,429</sup> Thus, the usually tightly controlled microglial activation may become self-amplifying and even neurotoxic.<sup>334</sup> Whether this dysregulation of microglial function plays a role in pneumococcal meningitis seems plausible but has not yet been investigated.

### Neuronal apoptosis

Neuronal damage is caused by the dual effects of an overwhelming inflammatory reaction and direct effects of bacterial toxins.<sup>430</sup> Though the hippocampus is not exposed to pneumococci or infiltrating leukocytes directly, it is surrounded by interstitial fluid which is contiguous with the CSF, allowing secreted bacterial toxins and immune system mediators to diffuse into the parenchyma.<sup>401,431</sup> Recent research with murine models has shown that pneumococcus-mediated hippocampal apoptosis occurs in at least two phases, separated by both time and mechanism.<sup>432,433</sup>

The early phase is initiated by the pneumococcal toxins H<sub>2</sub>O<sub>2</sub> and pneumolysin and results in caspase-independent apoptosis-like pyknotic cell death of both mature and immature neurons throughout the dentate gyrus of the hippocampus.<sup>401,432</sup> This is subsequently followed by caspase-dependent apoptosis of the immature neurons in the subgranular region of the dentate gyrus, triggered primarily by bacterial cell wall stimulation of leukocytes.<sup>413,432</sup>

Pneumolysin, which is also implicated in apoptosis of microglial and brain microvascular endothelial cells, was shown to colocalize to dying neurons in the dentate gyrus in a rabbit model of pneumococcal meningitis.<sup>185,401</sup> Animals infected with either pneumolysin- or H<sub>2</sub>O<sub>2</sub>-deficient pneumococci showed only partial attenuation of early neuronal apoptosis.<sup>401</sup> Additional blocking of H<sub>2</sub>O<sub>2</sub> in animals infected with pneumolysin-deficient pneumococci led to a marked further reduction of cell death, suggesting that H<sub>2</sub>O<sub>2</sub> and pneumolysin together are responsible for early hippocampal apoptosis.<sup>401</sup>

Because pneumococci do not express catalase, they are capable of producing high levels of H<sub>2</sub>O<sub>2</sub>, which can diffuse freely through the cellular membranes of target cells to damage intracellular structures.<sup>434</sup> H<sub>2</sub>O<sub>2</sub> triggers the release of Ca<sup>2+</sup> from the endoplasmic reticulum and its influx from the extracellular space. This results in a loss of mitochondrial membrane stability and in further increases of Ca<sup>2+</sup> and ROS production. Pneumolysin possibly contributes directly to mitochondrial permeabilization through pore formation,

after which apoptosis inducing factor (AIF) is cleaved from the mitochondrial membrane by calpain and cathepsin and is transported through the cytosol into the nucleus.<sup>435,436</sup> There, AIF causes chromatin condensation and large-scale DNA fragmentation, leading to cell death.<sup>437</sup> Paradoxically, AIF may also have antiapoptotic properties through the regulation of ROS through peroxide scavenging.<sup>437</sup>

The late, caspase-dependent phase of neuronal apoptosis is PCW and TLR2 dependent.<sup>400,432</sup> The observation that *in vitro* exposure of isolated cultured neurons to PCW products does not lead to cell death led to the hypothesis that late-phase apoptosis may be dependent on the host inflammatory response.<sup>432</sup> Neurons themselves do not express TLR2 or -4 and are not sensitive to exposure to the corresponding bacterial ligands. However, when cocultured with microglial cells, neurons revealed caspase-dependent, TLR-mediated late apoptosis when they were exposed to PCW or LPS.<sup>438,439</sup> Therefore, the inflammatory response of microglial cells and invading neutrophils may underlie the caspase-dependent hippocampal apoptosis during pneumococcal meningitis.

Several experimental treatments aimed at reducing hippocampal apoptosis have been studied in animal models of pneumococcal meningitis. Much attention has gone to nonbacteriolytic antibiotic therapies, such as rifampin, daptomycin, and clindamycin (see Targets for Adjunctive Therapy). In experimental rabbit models, rifampin given either alone or as a pretreatment before ceftriaxone resulted in a reduction of hippocampal apoptosis, though no reduction in mortality was observed.<sup>440-442</sup> Likewise, both daptomycin and clindamycin treatments yielded less hippocampal neuronal damage than that with ceftriaxone treatment in rabbit and rat models, respectively.<sup>443,444</sup> Finally, inhibition of MMP and TNF- $\alpha$  converting enzyme (TACE) was also shown to reduce cortical damage and neuronal apoptosis, as well as preserving learning performance in rats with pneumococcal meningitis.<sup>392</sup>

### Sepsis and hippocampal damage

Recent findings with experimental animal models have suggested that neuronal damage may also be caused by pneumococcal growth outside the CNS compartment.<sup>445</sup> Mice exposed intravascularly to purified PCW developed hippocampal apoptosis at 6 h postinoculation, an effect that was not reproduced in TLR2- or NOD2-deficient mice and was limited in mice overexpressing IL-10.<sup>332</sup> These findings suggest a possible third, earlier, IL-10-repressible mechanism of neuronal damage, which may precede pneumococcal invasion of the CNS.<sup>332</sup> Additionally, in the setting of experimental meningitis, bacteremia has been shown to contribute not only to increased hippocampal apoptosis but also to dysregulation of CBF autoregulation, reduced meningeal inflammation, and attenuated CSF pleocytosis.<sup>446,447</sup> The mechanisms involved remain largely unclear.

### Cochlear damage and hearing

Hearing loss is a common long-term complication in survivors of bacterial meningitis. Up to 30% of survivors of pneumococcal meningitis experience uni- or bilateral

hearing loss, which is often permanent and may be quite severe.<sup>147,448-450</sup> The underlying pathophysiology has been studied in several animal models.<sup>451-456</sup> Cochlear involvement may result from direct spread of pneumococcal infection from the meninges, CSF, and cochlear perilymphatic system.<sup>451,454,457</sup> Alternatively, a hematogenous route may occur following bacteremia or sepsis.<sup>454,457</sup>

The resulting cochlear infiltration of pneumococci and neutrophils results in a severe granulocytic inflammation of the perilymphatic spaces and in the release of proinflammatory cytokines and cytotoxic mediators.<sup>458</sup> Hearing loss was correlated with the level of CSF inflammation in a rabbit model of pneumococcal meningitis, and intrathecal administration of TNF- $\alpha$  alone was sufficient to induce cochlear injury similar to that observed with bacterial meningitis.<sup>451,459</sup> Furthermore, TLR and MyD88 knockout mice demonstrated less hearing loss following pneumococcal meningitis.<sup>460</sup> In a rat model, cochlear expression of iNOS and eNOS was upregulated following pneumococcal meningitis, and RNS-mediated cochlear damage could be attenuated both electrophysiologically and histopathologically by RNS scavengers.<sup>410,457,461</sup> Earlier studies with guinea pigs showed that local perfusion of the scala tympani with NO donor compounds resulted in cochlear damage and could be attenuated by NO inhibitors or O<sub>2</sub> scavengers.<sup>462</sup>

### Cerebrovascular complications

Cerebrovascular complications are very common during pneumococcal meningitis.<sup>2,9</sup> Arterial stroke occurs in up to 30% of patients, cerebral venous thrombosis in 9%, and intracerebral hemorrhage in up to 9%.<sup>6,147,463</sup> Autopsy studies in the 1930s through 1960s showed inflammatory infiltrations of cerebral arteries and veins.<sup>464-466</sup> Taking these together with angiographic descriptions of segmental arterial narrowing in patients with ischemic stroke complicating pneumococcal meningitis, the general assumption has been that infarctions during bacterial meningitis are caused by vasculitis.

In a recent human histopathological analysis of patients with pneumococcal meningitis, among whom half of patients had evidence of cerebral infarctions and 67% showed microhemorrhages, there was no evidence of large-vessel vasculitis.<sup>407</sup> Moreover, the observed small-vessel vasculitis did not colocalize with areas of infarction, and in this series, no evidence of disseminated intravascular coagulation in the systemic compartment was observed.<sup>407</sup> These results suggest the possibility of cerebral intravascular coagulation, independent of systemic coagulopathy or cerebral vasculitis, as the cause for both cerebral infarctions and hemorrhages.

The pathogenesis of cerebral infarction remains unclear and is the subject of ongoing research, which has focused largely on two areas: first, the dysregulation of the coagulation and fibrinolytic pathways, not only systemically but also locally, as exemplified by the upregulation of PAI-1 and elevated levels of prothrombin fragments F1 and -2 and soluble tissue factor in the CSF of patients with pneumococcal meningitis; and second, endothelial cell dysfunction, which may lead to localized swelling and release of procoagulant factors and proinflammatory cytokines. Also, endothelin, which is one of several potent vasoactive

peptides, has been shown to be elevated in CSF during acute stages of infection.<sup>245,406,467-470</sup> In a rat model, treatment with bosentan (an endothelin antagonist) normalized otherwise reduced CBF. Although endothelin inhibition lowered cortical necrosis, no effect on hippocampal damage was observed.<sup>470</sup>

## Targets for adjunctive therapy

### Inhibition of complement activation

Complement activation is crucial in the early phases of host defense against pneumococcal disease. Generally, complement activation leads to formation of a membrane pore (the MAC) in the pathogen, leading to cell lysis. However, complement components C3a, C4a, and C5a are cleaved in the activation of the complement cascade and serve as anaphylatoxins.<sup>471</sup> They recruit leukocytes to the site of infection, enhance neutrophil survival, and inhibit neutrophil oxidative burst.<sup>472-474</sup> In a murine sepsis model, a C1 inhibitor improved survival through complement inhibition.<sup>475</sup> In experimental pneumococcal meningitis, inhibition of C1 resulted in reduced meningeal inflammatory responses, decreased cytokine levels, decreased bacterial outgrowth, and improved survival in rats.<sup>476</sup> Interference in the final common complement pathway may present a promising future target for adjunctive therapy (Table 4).

### Inhibition of proinflammatory cytokines

TNF- $\alpha$  is essential for a robust inflammatory response but may also elicit inflammation-related complications.<sup>313,315</sup> Thalidomide is a TNF- $\alpha$  inhibitor which is used in the treatment of multiple myeloma.<sup>480</sup> In a rabbit model induced by intrathecally administered heat-killed pneumococci, intraperitoneally administered thalidomide was associated with decreased CSF TNF- $\alpha$  levels (but not IL-1 $\beta$  levels) and decreased CSF pleocytosis, but there was no effect on blood-brain barrier permeability.<sup>481</sup> When TNF- $\alpha$  was blocked by a TACE inhibitor in an experimental mouse model of pneumococcal meningitis, survival was increased in WT and TLR2-/- mice.<sup>482</sup> Thus, TACE inhibition may improve survival, even in a host with deficient TLR2 signaling.<sup>482</sup>

Blocking IL-6 intravenously in a rat model of pneumococcal meningitis reduced CSF pleocytosis and protein content.<sup>483</sup> Similar results were found with antibodies against IL-10 administered intravenously, which also decreased CSF IL-6 levels.<sup>335</sup> Administration of recombinant TGF- $\beta$ 2 intraperitoneally in the acute phase of pneumococcal meningitis in rats reduced the subarachnoid inflammatory response by inhibiting the increase in CBF and brain water content.<sup>338</sup>

**Table 4:** Therapeutic/adjuvant treatments in experimental settings.

Method of treatment	Target/treatment	Outcome	Reference
Inhibition of leukocyte migration	L-selectin/fucoidin	Lowers CSF pleocytosis and protein content, CBF, ICP, and cytokine production (IL-1 and TNF- $\alpha$ )	485, 486, 487, 488, 256, 344, 350
	ICAM-1/ABs	Reduced CSF leukocyte count, CBF, ICP, and brain edema	352
	CD-18/ABs	Reduction of CSF pleocytosis, blood-brain barrier permeability, and brain edema and increased survival	350
	G-CSF	(Pre)treatment with G-CSF leads to lower levels of CSF pleocytosis and proinflammatory cytokines	494, 495, 496
	P-selectin/pertussis toxin and E-selectin	Lowers CSF pleocytosis	500
Inhibition of pattern recognition receptors	ERK1/2/kinase inhibitor AG126	Decreased microglial production of proinflammatory cytokines and chemokines, pleocytosis, CBF, and brain edema	305
Inhibition of proinflammatory cytokines	TNF- $\alpha$ /thalidomide	Decreased TNF- $\alpha$ levels (but not IL-1 <sub>β</sub> levels), decreased CSF pleocytosis	477
	TNF- $\alpha$ /TACE	Increased survival in murine model in both WT and TLR2 knockout mice	482
	IL-6/ABs	Reduced CSF pleocytosis and CSF protein content in rat model	483
	IL-10/ABs	Reduced CSF pleocytosis, CSF protein content, and IL-6 level	335
Use of nonbacteriolytic antibodies	Daptomycin	Reduced levels of inflammatory cytokines and cortical damage	548, 549, 444
	Rifampin	Reduced bacterial protein synthesis, lowered mortality in murine model	551
	Moxifloxacin	Reduced release of PCW products, inflammation, and neuronal damage in combination with ceftriaxone	441, 552, 442
Radical scavenging	iNOS inhibitors/NAC	Reduced bacterial cell wall components LTA and TA in CSF in rabbits	551
		No reduction in mortality over cephalosporin therapy	555
Inhibition of caspases	iNOS inhibitors/NAC	Reduction of proinflammatory cytokine production, cortical damage, CSF pleocytosis, and hearing loss	556, 557, 397, 461, 558
	Caspase-3/BDNF	Reduction of neuronal apoptosis	505, 506
Inhibition of complement	All caspases/BAF	Reduction in cognitive decline	503
	C1	Reduction in meningeal inflammation response	476
Inhibition of MMPs	BB-94	Reduction of blood-brain barrier permeability, lowers ICP	389
	GM6001	Decreased TNF- $\alpha$ levels, reduction of neuronal apoptosis	478
	MMP and TACE/TNF484	Reduction of cortical necrosis No reduction of hippocampal apoptosis	479
	MMP and TACE/BB-1101	Reduction of both cortical necrosis and hippocampal apoptosis, preserved learning performance	392

### **Inhibition of pattern recognition receptors**

Inhibiting TLR signaling with the aim of decreasing subsequent cytokine responses presents a promising strategy. The kinase inhibitor tyrphostin AG126 was shown to inhibit phosphorylation of the signaling molecule extracellular signal-regulated kinase 1/2 (ERK1/2) in microglial cells.<sup>305</sup> ERK1/2 is activated in blood monocytes in response to LPS through activation of CD14 and TLR4.<sup>484</sup> In microglial cells, treatment with this kinase inhibitor resulted in decreased production of proinflammatory cytokines and chemokines.<sup>305</sup> When the kinase inhibitor was administered intraperitoneally in a mouse pneumococcal meningitis model, leukocyte recruitment to the CSF, CBF, brain edema, and TNF- $\alpha$  production were reduced.<sup>305</sup> This provided evidence that blocking the TLR signaling pathway may reduce the severity of disease in pneumococcal meningitis.

### **Inhibition of leukocyte influx into the CNS**

One of the strategies to prevent brain damage is to limit leukocyte recruitment to the CSF or to increase leukocyte apoptosis. Recruitment of leukocytes (mainly polymorphonuclear leukocytes [PMNs]) to the subarachnoid space results in the clearance of bacteria, which is accompanied by the production of several toxic mediators that may induce damage not only to the bacteria but also to the brain.

The first step in extravasation of PMNs involves binding of the leukocytes to selectins on the vessel endothelium. Fucoidin is a polysaccharide that blocks the leukocyte receptor L-selectin. Intravenous treatment with fucoidin in several animal models of pneumococcal meningitis reduced CSF pleocytosis, in association with reduced CSF protein content, modestly decreased CSF lactate, and decreased CBF and ICP, without influencing blood-brain barrier permeability, cerebral edema, and outcome.<sup>256,485,486</sup> Furthermore, fucoidin prevented the increase in CSF TNF- $\alpha$  and IL-1 levels in response to intrathecally administered PCW in rabbits; however, when live bacteria were administered intrathecally and the rabbits were treated with ampicillin, fucoidin had no effect on cytokine production.<sup>487</sup> A similar study without antibiotic treatment reported a reduction in IL-1 and an increase in CSF IL-8 in fucoidin-treated rabbits with pneumococcal meningitis.<sup>344</sup> In contrast to these studies, in a rat model of pneumococcal meningitis, fucoidin treatment led to decreased survival.<sup>488</sup> The leukocyte concentration in CSF was lower in fucoidin-treated rats, but systemic leukocytosis was increased, as was systemic bacterial outgrowth. However, rats in this study were pretreated with fucoidin, which may have led to the differences in the systemic immune response.<sup>488</sup>

After binding of leukocytes to selectins, firm adhesion to the vascular endothelium is mediated by ICAM-1. In a rat model of meningitis with PCW component-induced inflammation, antibodies against ICAM-1 reduced CSF leukocyte counts, CBF, ICP, and brain edema in the first 6 h.<sup>352</sup> In experimental pneumococcal meningitis, an intravenously administered antibody against CD18, a subunit on leukocytes for binding ICAM-1, induced either by live bacteria or by PCW, reduced CSF leukocyte counts, blood-brain barrier permeability, and brain edema and increased survival.<sup>350</sup> However, anti-CD18

antibodies tended only to inhibit CSF leukocyte counts in a rabbit model of PCW-induced inflammation.<sup>489</sup>

Vascular endothelial growth factor (VEGF) is a peptide involved in angiogenesis, but it has also been described to function as a macrophage and granulocyte chemoattractant.<sup>490</sup> Levels of VEGF were associated with CSF leukocyte counts in experimental meningitis induced by heat-killed pneumococci.<sup>491</sup> However, blocking of VEGF in rabbits with pneumococcal meningitis did not reduce the extent of brain edema, leukocyte influx, or blood-brain barrier permeability.<sup>491</sup>

A different approach involves the induction of an increased systemic proliferation of leukocytes, which would lead to better control of systemic infection and, subsequently, to enhanced control of CNS infection. In patients with meningitis, granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) have been found to be elevated in the CSF.<sup>492,493</sup> In these studies, G-CSF and M-CSF levels correlated with CSF leukocytosis.<sup>493</sup> In mice with pneumococcal meningitis, expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the brain was increased.<sup>319</sup> Rabbits pretreated with G-CSF intravenously 1 h before intrathecal inoculation with *S. pneumoniae* showed increased peripheral but not subarachnoid leukocytosis and increased CSF levels of TNF and IL-1 but no reduction of subarachnoid bacterial outgrowth or neuron-specific enolase, an indicator of neuronal cell damage.<sup>316</sup> A similar experiment showed no influence on subarachnoid bacterial killing, but systemic pleocytosis and bacterial killing were increased.<sup>494</sup> CSF leukocytosis and protein content and levels of IL-8, TNF- $\alpha$ , and IL-1 $\beta$  were decreased in G-CSF-pretreated animals, indicating a decreased subarachnoid inflammatory response.<sup>494</sup> Similar results were found in a study with rats.<sup>495</sup> However, late administration of G-CSF (28 h after infection) did not have any influence on disease parameters.<sup>495</sup> In 22 patients with pneumococcal meningitis, adjunctive treatment with recombinant G-CSF was performed together with standard treatment with ceftriaxone and dexamethasone. G-CSF was continued for 4 days unless leukocyte counts exceeded  $40 \times 10^9$  cells/liter. Lactate and glucose levels returned to normal more quickly in G-CSF-treated patients than in historical controls, and no adverse events were recorded. However, this was not a randomized controlled study.<sup>496</sup>

Another approach to limit neutrophil-mediated damage in pneumococcal meningitis is to induce apoptosis in neutrophils by use of roscovitine. Mice treated with a combination of antibiotics and roscovitine showed increased resolution of inflammation, decreased cerebral hemorrhages, and faster recoveries.<sup>497</sup>

In meningitis caused by *Cryptococcus neoformans*, the fungal capsular polysaccharide glucuronoxylomannan (GMX) inhibited leukocyte extravasation despite high IL-8 levels in the CSF.<sup>498</sup> The mechanisms by which GMX inhibits leukocytosis are unknown; however, when GMX was administered intravenously in an experimental rabbit model of meningeal inflammation with heat-killed pneumococci, CSF TNF- $\alpha$  levels and leukocytosis decreased, in association with reduced brain edema and inflammation on brain histopathology.<sup>499</sup>

A similar mechanism has been described for pertussis toxin, which interferes with the binding of PMNs to P-selectin and E selectin, the first steps of diapedesis. Intravenous treatment with pertussis toxin in a rabbit model of meningeal inflammation induced by heat-killed pneumococci altered CSF pleocytosis compared to that in untreated animals.<sup>500</sup>

### **Inhibition of Caspases**

Caspase activation has been implicated in the activation of proinflammatory cytokines as well as in the mediation of programmed cell death of cerebral endothelial cells and neurons in the hippocampal dentate gyrus.<sup>282,400,432</sup> Experimental models of pneumococcal meningitis using mice deficient in caspase-1 or after pharmacological blocking of caspase-1 demonstrated lower levels of IL-1 $\beta$  and subsequent diminished proinflammatory cytokine production, as well as fewer meningitis-induced complications.<sup>282</sup> The protective effect of caspase-3 inhibition on the development of neuronal damage was demonstrated in a rat model of pneumococcal meningitis and was independent of cytokine modulation.<sup>501</sup> Also, the broad-spectrum caspase inhibitor z-VAD-fmk was shown to reduce hippocampal neuronal apoptosis in a rabbit model of pneumococcal meningitis compared to that in untreated controls.<sup>502</sup> Moreover, rats inoculated with group B streptococci demonstrated less cognitive decline following adjunctive treatment with the pan-caspase inhibitor bocasparyl (OMe)-fluoromethylketone.<sup>503</sup> An interesting observation is that exogenous administration of brain-derived neurotrophic factor (BDNF), which has been shown to block caspase-3, reduced neuronal apoptosis in both rat and murine models.<sup>504-506</sup> BDNF was found to be upregulated naturally during bacterial meningitis and after treatment with antibiotics with adjunctive dexamethasone yet lowered during standard antibiotic treatment, suggesting a possible mechanism of corticosteroid therapy.<sup>507</sup> So far, no clinical trials have been performed with caspase inhibitors as adjunctive therapy.

### **Adjunctive Dexamethasone Therapy**

Dexamethasone is a widely used anti-inflammatory drug. The mechanisms by which dexamethasone inhibits inflammation are not clear, but it decreases proinflammatory cytokine production in monocytes, dendritic cells, astroglial cells, and neutrophils, increases the production of anti-inflammatory cytokines such as IL-10, inhibits ROS production by leukocytes, and decreases leukocyte adherence.<sup>241,304,508-511</sup> Dexamethasone acts on multiple molecules of the TLR downstream signaling cascade, including TAK-1, ERK1/2, MAPK, NF- $\kappa$ B, and STAT3.<sup>512,513</sup> Astroglial cells stimulated with PCW components produced decreased amounts of NO and TNF- $\alpha$  when they were treated with dexamethasone.<sup>304,514,515</sup> Brain microvascular endothelial cells also showed reduced levels of TNF- $\alpha$  and IL-1 and decreased expression of ICAM-1.<sup>241</sup> On the molecular level, in peripheral blood mononuclear cells dexamethasone was shown to inhibit *S. pneumoniae*-induced I $\kappa$ B $\kappa$  phosphorylation and degradation and binding of NF- $\kappa$ B to DNA, both of which are downstream effector mechanisms of TLR signaling.<sup>516</sup> Dexamethasone induced increased levels of I $\kappa$ B $\kappa$  mRNA, which may bind and inhibit the

p65 subunit of NF- $\kappa$ B. These effects resulted in decreased IL-8 production by peripheral blood mononuclear cells.<sup>516</sup>

In experimental pneumococcal meningitis, adjunctive dexamethasone reduced TNF- $\alpha$ , lactate, and NO levels when it was administered together with antibiotics. Furthermore, dexamethasone decreased ICP, brain edema, and CSF pleocytosis in rats with PCW-induced meningeal inflammation.<sup>517-520</sup> In a rabbit model, neuron-specific enolase, a marker of overall neuronal damage, was reduced in animals treated with ceftriaxone and dexamethasone compared to those treated with ceftriaxone alone, though an increase in hippocampal apoptosis was also observed.<sup>521</sup> Moreover, in rats treated with adjunctive dexamethasone, the observed increase in hippocampal apoptosis was accompanied by impaired learning performance, and uninfected rats treated with dexamethasone also demonstrated increased hippocampal damage.<sup>522,523</sup> In an analysis of several prospective multicenter trials in which patients with bacterial meningitis were treated with either adjunctive dexamethasone or placebo, the use of dexamethasone was not associated with cognitive impairment.<sup>8,409</sup>

Adjuvant treatment with dexamethasone resulted in a reduction of hearing loss in rabbit and gerbil models of pneumococcal meningitis but did not have a significant effect in infant rats.<sup>524-526</sup> A recent meta-analysis of human trials evaluating adjuvant dexamethasone treatment suggested that dexamethasone may reduce hearing loss among survivors.<sup>16</sup> Clearly, further trials are necessary to assess the effects of dexamethasone on cochlear injury and hearing loss.

In children with bacterial meningitis, CSF TNF- $\alpha$  and IL-1 levels were decreased if patients had been treated with adjunctive dexamethasone therapy.<sup>527</sup> In a large randomized controlled trial in Vietnam investigating the efficacy of dexamethasone addition to conventional antibiotic regimens in adults with bacterial meningitis, CSF samples from a large group of patients were examined.<sup>528</sup> For 195 of a total of 341 patients included in this study, CSF was available at baseline (when therapy was started) and at a follow-up puncture 1 to 4 days later. Of these 195 patients, 88 had received dexamethasone along with antibiotics starting at baseline, and 107 received placebo and antibiotics. For 24% of patients, *S. pneumoniae* was confirmed as the causative agent by CSF culture. Other causative agents included *S. suis* (43%) and *N. meningitidis* (8%). CSF samples from these patients were analyzed for IL-6, IL-8, IL-10, IL-12, IL-1 $\beta$ , and TNF- $\alpha$ . Cytokine levels in CSF from the first lumbar puncture at baseline were similar between both groups. Dexamethasone treatment reduced the levels of IL-6 and IL-8 and increased the levels of IL-10 (median, 37 pg/ml versus 33 pg/ml; P value = 0.01) in the CSF at follow-up compared to those with placebo.<sup>528</sup> Levels of IL-12, IL-1 $\beta$ , and TNF- $\alpha$  were similar between both groups at follow-up. In addition to the differences in anti-inflammatory cytokine profiles, opening pressure of the follow-up lumbar puncture was reduced and the CSF glucose level and CSF/plasma glucose ratio were restored sooner in patients receiving dexamethasone. CSF lactate and protein levels as well as leukocyte counts were similar at follow-up in the dexamethasone and placebo groups.<sup>528</sup>

Many randomized clinical trials of dexamethasone for treatment of bacterial meningitis have been performed, but the results have remained somewhat ambiguous.<sup>4,11,15,16,427</sup> An individual patient data meta-analysis of 5 large recent trials showed no effect of dexamethasone.<sup>16</sup> A prospective cohort study showed a decrease in mortality from 30 to 20% for adults with pneumococcal meningitis after nationwide implementation of dexamethasone therapy in the Netherlands.<sup>17</sup> Dexamethasone treatment has been implemented as routine therapy for patients with suspected or proven pneumococcal meningitis in many countries.<sup>2,529</sup>

### Adjunctive Glycerol Therapy

Glycerol (glycerine 1-2-3-propanetriol), a hyperosmolar compound, is an essential component of cell membranes. It has been used in neurosurgery, neurology, and ophthalmology to decrease raised tissue pressures.<sup>530-535</sup> Toxicological data show that it is safe and is associated with few, rare, mild, mostly gastrointestinal side effects.<sup>536</sup> Furthermore, glycerol is inexpensive and readily available, facilitating widespread implementation if it is effective. The effects of glycerol in the neurological/neurosurgical setting have been hypothesized to lie in the resulting increase of plasma osmolality, which was shown to reduce the excretion of CSF by some 20 to 30%, leading to increased cerebral blood flow and improvement of brain oxygenation.<sup>537</sup> For acute stroke, glycerol has been shown to confer only a short-term advantage, without demonstrating benefits in the long-term outcome.<sup>534</sup>

The first clinical trial evaluating glycerol as a potential adjuvant treatment for bacterial meningitis was conducted with 122 Finnish children with bacterial meningitis and suggested a reduction in hearing impairment as well as in long-term neurological sequelae.<sup>538</sup> More recently, a large South American trial using adjuvant glycerol for the treatment of children with bacterial meningitis provided additional support for its efficacy in the reduction of neurological sequelae, although hearing loss and mortality were not diminished.<sup>539,540</sup> However, reliable interpretation of the findings was compromised by several methodological problems.<sup>541</sup> Nevertheless, the results fueled additional trials, most recently a study in Malawi where 256 adults with bacterial meningitis were randomized to receive either placebo or glycerol as adjuvant treatment.<sup>542</sup> The trial was halted prematurely when an interim analysis after 100 deaths showed increased death in the glycerol group.<sup>542</sup> The discrepancies in the various studies most likely lie in the variation in study populations, such as age, comorbidity, and causative pathogen, as well as in the variation in treatment regimens and methods of assessing outcome parameters.

Relatively few animal studies have been performed with glycerol, and they have not been able to demonstrate clinical or histological benefits of adjuvant glycerol therapy.<sup>543</sup> Moreover, in a rabbit model, pneumococcal meningitis treated with glycerol alone was associated with an increased level of hippocampal neuronal apoptosis.<sup>544</sup> A study in which healthy mice were treated with high doses of glycerol (much higher than trial dosages) showed that treatment was associated with the occurrence of seizures, which were also seen more in the glycerol-

treated patients in the Malawi trial.<sup>542,545</sup> This finding was not in concurrence with the South American trial and may be explained by either the shorter glycerol regimen (2 days instead of 4 days) or increased disease severity in the Malawi group.<sup>542</sup>

Though glycerol seemed attractive as a potential adjuvant treatment for bacterial meningitis, the lack of effectiveness in experimental models, combined with its harmful effects in the Malawi trial, question the value of additional studies on adjunctive glycerol for the treatment of adult meningitis.<sup>546</sup>

### Nonbacteriolytic Antibiotics

The massive inflammatory response following pneumococcal meningitis has been shown to play a key role in the development of brain damage and subsequent poor outcomes.<sup>547</sup> In part, the inflammatory response is determined by bacterial lysis products, as shown in experimental pneumococcal meningitis models where inoculation with PCW led to massive inflammation and neuronal damage.<sup>250,254</sup> Although bacteriolytic antibiotic regimens may limit the overall amount of release of bacterial products, temporary increases in the release of bacterial components have been documented following treatment.<sup>251</sup> These observations have fueled the study of nonbacteriolytic antimicrobials as future therapy options.

Specifically, recent research of nonbacteriolytic antimicrobials has focused on the potential use of daptomycin, rifampin, and moxifloxacin. Daptomycin, a lipopeptide antibiotic, has been shown to effectively clear *S. pneumoniae* in experimental rat, mouse, and rabbit meningitis models.<sup>444,548</sup> Treatment with daptomycin led to lower levels of inflammatory cytokines and possibly to less cortical brain damage and neuronal apoptosis than those for treatment with ceftriaxone alone.<sup>444,549</sup> Although adjunctive treatment with dexamethasone did not result in a reduction of overall pneumococcal clearance from the CSF, daptomycin penetration into the inflamed meninges was reduced in the presence of adjunctive corticosteroid therapy.<sup>550</sup>

Rifampin inhibits bacterial protein synthesis and was shown to lead to diminished levels of PCW product release *in vitro*.<sup>551</sup> In a murine model of pneumococcal meningitis, a decrease in mortality was observed in mice treated with rifampin versus ceftriaxone, an effect which was most notable during the first hours following antibiotic therapy.<sup>552</sup> Moreover, recent studies with a rabbit model demonstrated that short-term pretreatment with rifampin before ceftriaxone reduced the release of PCW products, inflammation, and neuronal damage compared to those with treatment with ceftriaxone alone.<sup>440-442</sup>

In a rabbit model of pneumococcal meningitis, treatment with moxifloxacin, a relatively novel quinolone, was shown to result in lower levels of the proinflammatory cell wall components LTA and TA in CSF than those obtained by treatment with ceftriaxone.<sup>551</sup> Pneumococcal clearance from the CSF was comparable after treatment with either moxifloxacin or ceftriaxone, and drug levels were not reduced following adjunctive treatment with dexamethasone.<sup>553,554</sup> However, in a murine model, moxifloxacin failed to reduce mortality compared to standard cephalosporin therapy.<sup>555</sup>

### Radical Scavenging

Investigations of potential adjunctive antioxidant therapies have been limited to animal studies and so far lack sufficient evidence for clinical testing. A recent comprehensive review by Klein et al. summarizes the efforts and findings in this area of potential treatment options.<sup>397</sup> Among the most promising candidates for future clinical applications are the iNOS inhibitors, the peroxynitrite scavenger Mn(III)tetrakis(4-benzoic acid)-porphyrin (MnTBAP), uric acid, alpha-pheny-tert-butyl-nitron (PBN), and N-acetyl-L-cysteine (NAC).<sup>397</sup> In spite of some beneficial effects, such as reductions of proinflammatory cytokine pleocytosis, reductions in cortical damage, attenuated CSF pleocytosis, and a diminished incidence of hearing loss, adverse outcomes have been reported, such as impaired bacterial killing, increased neuronal apoptosis, and impaired learning function.<sup>461,556-558</sup> Of all potential antioxidant agents, only NAC is currently used routinely in clinical practice (for the treatment of acetaminophen intoxications), and therefore it is a likely candidate for evaluation in a clinical setting.

### Conclusions

Despite significant advances in treatment and vaccinations, pneumococcal meningitis remains one of the most important infectious diseases worldwide and continues to be associated with high mortality and morbidity. The growing emergence of drug resistance as well as shifts in serotype incidence is fueling further development of novel antibiotic and adjuvant treatment strategies. In addition to the widespread introduction of dexamethasone, other options for adjuvant drugs may lie in modulating ROS/RNS-mediated damage, in caspase inhibition, or in drugs targeting specific mediators in the inflammatory, complement, or coagulation cascades. Extensive research in this area is being performed using experimental animal meningitis models, though so far no clinical treatment trials with humans have been performed. Although the limitations of animal models of meningitis lie in the great variability between species, inoculation methods, and ages of infected animals, experimental medicine continues to provide the backbone for both intervention and pathophysiology studies and will hopefully pave the way to new knowledge and treatment of this deadly disease.

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# Characterization of a pneumococcal meningitis mouse model

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## Abstract

Background: *S. pneumoniae* is the most common causative agent of meningitis, and is associated with high morbidity and mortality. We aimed to develop an integrated and representative pneumococcal meningitis mouse model resembling the human situation.

Methods: Adult mice (C57BL/6) were inoculated in the cisterna magna with increasing doses of *S. pneumoniae* serotype 3 colony forming units (CFU; n = 24, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup> and 10<sup>7</sup> CFU) and survival studies were performed. Cerebrospinal fluid (CSF), brain, blood, spleen, and lungs were collected. Subsequently, mice were inoculated with 10<sup>4</sup> CFU *S. pneumoniae* serotype 3 and sacrificed at 6 (n = 6) and 30 hours (n = 6). Outcome parameters were bacterial outgrowth, clinical score, and cytokine and chemokine levels (using Luminex<sup>®</sup>) in CSF, blood and brain. Meningeal inflammation, neutrophil infiltration, parenchymal and subarachnoidal hemorrhages, microglial activation and hippocampal apoptosis were assessed in histopathological studies.

Results: Lower doses of bacteria delayed onset of illness and time of death (median survival CFU 10<sup>4</sup>, 56 hrs; 10<sup>5</sup>, 38 hrs, 10<sup>6</sup>, 28 hrs. 10<sup>7</sup>, 24 hrs). Bacterial titers in brain and CSF were similar in all mice at the end-stage of disease independent of inoculation dose, though bacterial outgrowth in the systemic compartment was less at lower inoculation doses. At 30 hours after inoculation with 10<sup>4</sup> CFU of *S. pneumoniae*, blood levels of KC/CXCL1, IL6, MIP-2/CXCL2 and IFN-  $\gamma$  were elevated, as were brain homogenate levels of CXCL1, CXCL2, IL-6, IL-1 $\beta$  and RANTES/CCL5. Brain histology uniformly showed meningeal inflammation at 6 hours, and, neutrophil infiltration, microglial activation, and hippocampal apoptosis at 30 hours. Parenchymal and subarachnoidal and cortical hemorrhages were seen in 5 of 6 and 3 of 6 mice at 6 and 30 hours, respectively.

Conclusion: We have developed and validated a murine model of pneumococcal meningitis.

## Introduction

Bacterial meningitis is a life threatening infectious disease of the central nervous system (CNS). The annual incidence is estimated to be up to 2.6 to 6.0 cases per 100 000 in Europe and may be up to ten times higher in developing countries.<sup>1,2</sup> The most common pathogen beyond the neonatal period is *Streptococcus pneumoniae*, causing 70% of cases.<sup>1,3</sup> Despite advances in medical care, mortality from pneumococcal meningitis remains between 16% and 37% and neurological sequelae affect 30-52% of survivors.<sup>4-6</sup> There is a continuing need for the development of new treatment strategies.

Complications associated with pneumococcal meningitis include cerebral infarction, hemorrhages, motor and sensory deficit, seizures, memory and learning impairments, and hearing loss.<sup>3,7,8</sup> Autopsy studies of patients who died following pneumococcal meningitis revealed cerebral edema, cerebral infarctions and hemorrhages, apoptosis and necrosis of the hippocampal dentate gyrus.<sup>9-11</sup> Many of these pathological features have been reproduced in animal models, which provide the setting for novel drug development and pathophysiological studies.<sup>12,13</sup>

Several murine models have been developed, using intracerebral, intraperitoneal, intravenous, intranasal or intracisternal inoculation methods, and have recently been reviewed.<sup>12,14-19</sup> Problems with reproducibility, limited disease progression or iatrogenic structural damage, combined with a need for a single model in which most pathological features seen in human pneumococcal meningitis can be measured, has fueled the development of new animal models. Here we describe the development of an adult mouse model of pneumococcal meningitis in which many of the human pathological features are demonstrated.

## Methods

A clinical isolate of *S. pneumoniae* serotype 3 was obtained from ATCC (catalog number 6303), and was grown to mid log phase in 4 hours at 37°C in Todd-Hewitt broth supplemented with 0.5% yeast extract. At an OD<sub>620</sub> of 0.8 to 1.0 the *S. pneumoniae* were centrifuged and washed twice by resuspension in sterile 0.9% NaCl and recentrifugation. Finally, the bacteria were resuspended in sterile NaCl 0.9% to yield an approximate concentration of  $1 \times 10^9$  colony forming units (CFU)/ml. The exact number of CFUs was subsequently determined for inoculates by serial dilution method and on blood agar plates (overnight at 37°C).

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam. To determine the inoculation dose and optimal time points of sacrifice, 24 8-10 week old male C57BL/6 mice (Charles River Laboratories, Germany) received 0.1 mg/kg s.c. buprenorphine and short-term anesthesia using 1.5-2.0% isoflurane during inoculation. The mice were divided into 4

groups, each receiving a different concentration of bacterial inoculum ( $10^4$ ,  $10^5$ ,  $10^6$  and  $10^7$  CFU *S. pneumoniae* per mouse;  $n = 6$  per dose). Inoculation was conducted by injecting 10  $\mu$ L of bacterial suspension into the cisterna magna using a 32-gauge needle. All animals were evaluated directly following inoculation and subsequently at 4-hour intervals. The following scoring was used (Table 1): range: 0-41 pts; each scoring parameter ranging from 0, corresponding to no deficit, to a variable maximum score. The maximum score was determined by the estimated contribution of the variable to overall health of the mouse): weight loss (0-4 pts), activity (0-4 pts), time to return to upright position (0-6 pts), state of skin/fur (0-3 pts), posture (0-2 pts), eye discharge or protrusion (0-4 pts), respiration rate (0-4 pts), irregular/labored breathing (0-4 pts), epilepsy, limb paresis or ataxia (0-10pts). The clinical course was divided into a pre-symptomatic period (from time of inoculation until clinical score  $\leq 10$ ) and symptomatic period (clinical score  $> 10$  until death/sacrifice). Survival studies were performed and cerebrospinal fluid (CSF), brain, blood, spleen, and lungs were collected post mortem. After determining inoculation dose and time-points of sacrifice, the model was further characterized using 12 additional mice inoculated with  $10^4$  CFU *S. pneumoniae* serotype 3 and sacrificed at 6 ( $n = 6$ ) and 30 hours ( $n = 6$ ).

Bacterial titers were determined in samples of lung, brain, and spleen (diluted 1:4 in sterile NaCl 0.9% and homogenized). Blood was heparinized in a 1:4 dilution and CSF was diluted 1:100 in sterile NaCl 0.9%. All bacterial titers were determined by plating serial dilutions on blood agar plates and incubating overnight at 37 C.

Cytokine and chemokine measurement were performed on the left cerebral hemisphere diluted 1:4 in sterile NaCl 0.9%, homogenized and lysed in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM  $MgCl_2(H_2O)_6$ , 1 mM  $CaCl_2(H_2O)_2$ , 1% Triton, AEBSF 4  $\mu$ g/ml, EDTA-NA2 50  $\mu$ g/ml, pepstatin 10 ng/ml, leupeptin 10 ng/ml, pH 7.4). Samples of brain homogenate, serum and CSF were then centrifuged and supernatant stored at -80 C. Cytokine concentrations were determined with luminex<sup>®</sup> technology using a mouse cytokine and chemokine Bioplex kit (Bio-Rad Laboratories, Veenendaal, The Netherlands).

All mice were perfused by cardiac puncture with PBS prior to harvesting tissue. Histopathology was performed on the right cerebral hemisphere fixed in 4% paraformaldehyde and paraffin embedded. Coronal 10  $\mu$ m sections of the entire hemisphere were cut for subsequent staining. Hematoxylin and eosin (HE) and Nissl staining were performed to visualize hemorrhages, cortical necrosis, vasculitis and abscess formation. To determine neuronal apoptosis in the dentate gyrus of the hippocampus, four 10  $\mu$ m sections of the anterior, middle and posterior portion of the hippocampus were stained with Caspase-3 antibodies (polyclonal rabbit-anti-mouse, 1:100; Cell Signaling, Danvers, MA). In each section, the total number of caspase-3 positive cells was counted in both the dentate gyrus (DG) and cornu ammonis (CA) regions. Scoring was independently conducted by two investigators. Microglial activation was evaluated by immunohistochemistry using Iba-1 antibody (polyclonal rabbit-anti-mouse, 1:2000; ABcam, Cambridge, UK) staining of frontal lobe 10  $\mu$ m sections. No quantitative analysis was performed.

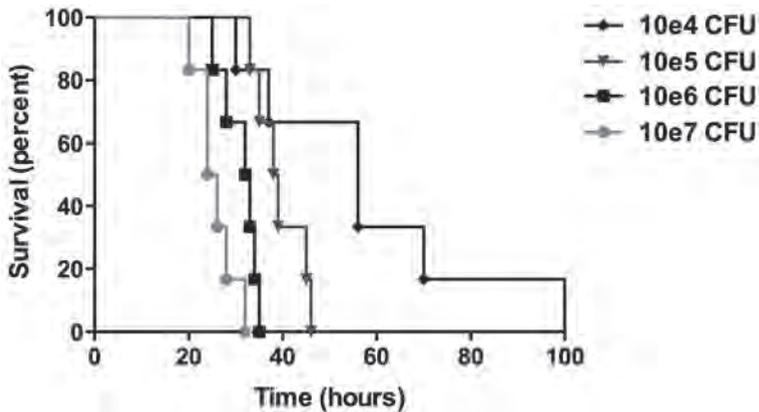
**Table 1:** Clinical score parameters, assessed values and weighted scores.

Parameter	Value	Weighted score	Maximum score
Weight loss from baseline	5%	0	
	10%	1	
	15%	2	
	20%	3	
	25%	4	4
Activity	normal	0	
	increased/decreased	1	
	mildly diminished	1	
	diminished	2	
	severely diminished	3	
Time to return to upright	normal	0	
	position upright < 5 sec	2	
	upright < 30 sec	4	
	no turn upright	6	6
Coat	normal	0	
	diminished grooming	1	
	soiled	1	
Posture	piloerection	1	3
	normal	0	
	slight hunched back	1	
Eyes	severely hunched back	2	2
	normal	0	
Respiration rate (per min)	protruding	1	
	sunken eyes	1	
	closed eyelids	1	
	discharge	1	4
	> 150	0	
Breathing	< 150	1	
	< 100	2	
	< 75	3	
	< 50	4	4
Neurologic exam	irregular	2	
	laboured	2	4
Total	normal	0	
	ataxia	2	
	limb paresis/paralysis	2	
	epileptic seizure	2	
Total	status epilepticus	6	10
			41

Comparisons of cytokine levels between groups were calculated using the Mann-Whitney U test. A Kruskal-Wallis one-way ANOVA was used to compare clinical scores of pre-symptomatic and symptomatic periods. Histopathological scores of neuronal apoptosis were compared using Student's *t*-test. For all analyses a p-value < 0.05 was considered significant.

## Results

Mortality occurred in nearly all inoculated mice (Figure 1); one mouse inoculated with  $10^4$  CFU survived beyond the study window of 216 hours after inoculation and was sacrificed. The median survival time was dose dependent ( $10^4$  CFU, 56 hrs;  $10^5$ , 38 hrs,  $10^6$ ; 28 hrs;  $10^7$ , 24 hrs). To approximate a physiological setting, we selected  $10^4$  CFU as the lowest concentration of bacterial inoculum in which most animals would die if left untreated. Furthermore, for future experimentation we chose 30 hours post inoculation as the latest time point for sacrifice, at which all animals were still alive and the natural course of the infection could be followed as long as possible.



**Figure 1:** Kaplan-Meier survival curve. Four groups of 6 mice inoculated via direct intracisternal injection with  $10^4$ ,  $10^5$ ,  $10^6$  and  $10^7$  CFU of *S. pneumoniae* per mouse respectively.

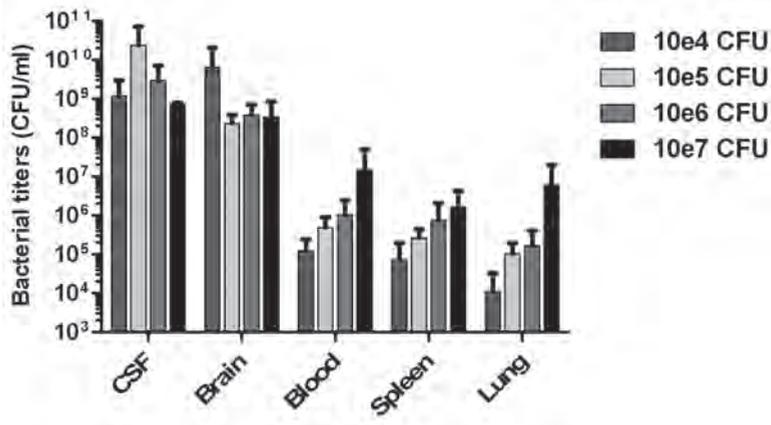
Clinical scoring was performed on all mice in the survival study. The average duration of the pre-symptomatic period (clinical score  $\leq 10$ ) was dose dependent, and increased approximately 1.5-fold with each successive 10-fold increase of bacterial inoculum concentration. The duration of the symptomatic period did not differ significantly between inoculation doses (mean, 11.7 hrs, SD 4.8; Table 2).

Bacterial meningitis was confirmed in all 23 mice in the survival study by way of culture of CSF and brain homogenate following death or sacrifice. The average pneumococcal concentration in the CSF and brain homogenates was  $2.0 \times 10^9$  CFU/ml and  $7.9 \times 10^8$  CFU/ml respectively. Bacterial titers in the CNS compartment (CSF and brain) did not increase with higher inoculation doses (Figure 2). In comparison with the CNS compartment, in the systemic compartment (blood, spleen, lung) bacterial concentrations were much lower (means  $1.0 \times 10^6$ ,  $4.0 \times 10^5$  and  $2.0 \times 10^5$  CFU/ml, respectively), and an increasing bacterial titer was observed with each successive 10-fold increase of bacterial inoculum concentration.

**Table 2:** Duration of pre-symptomatic (time from inoculation to clinical score  $\leq 10$ ) and symptomatic (time from clinical score  $> 10$  to death/sacrifice) periods.

Inoculation dose (CFU/mouse)	10 <sup>e4</sup>	10 <sup>e5</sup>	10 <sup>e6</sup>	10 <sup>e7</sup>	p-value
Pre-symptomatic period (hrs/st.dev)	40.7 (14.9)	26.1 (6.1)	18.7 (3.9)	12.0 (2.1)	0.001
Symptomatic period (hrs/st.dev)	9.1 (3.2)	13.0 (6.3)	10.8 (3.6)	13.6 (5.3)	0.557

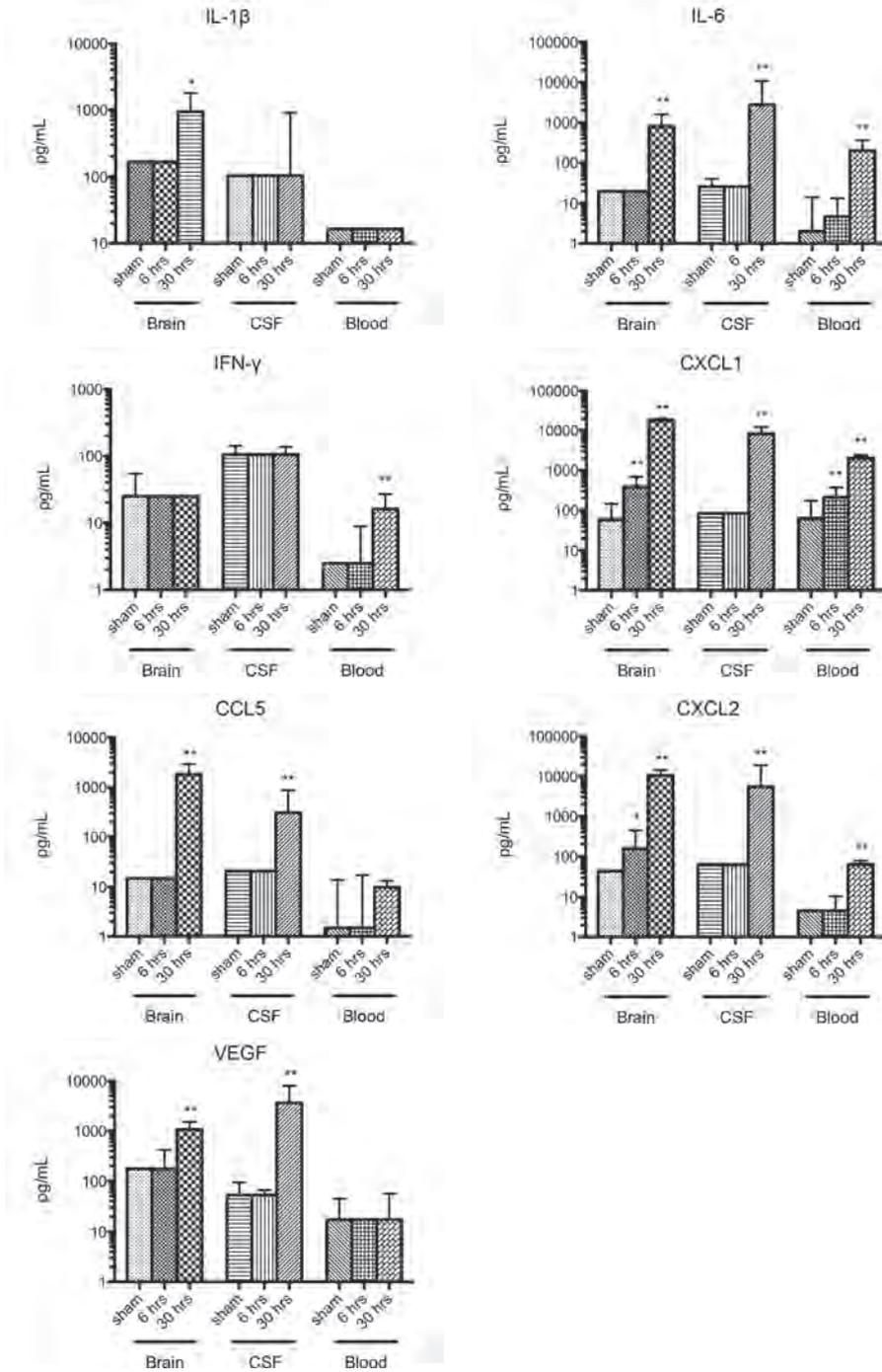
Mean durations of the pre-symptomatic and symptomatic periods (expressed in hours) of the mice of the survival study. Mice were divided into 4 groups and inoculated with 10<sup>4</sup> (5 mice, 1 mouse was excluded from analysis due to lack of any symptoms due to failed inoculation), 10<sup>5</sup> (6 mice), 10<sup>6</sup> (6 mice) and 10<sup>7</sup> CFU (6 mice) *S. pneumoniae* per mouse. Comparison of the clinical scores of pre-symptomatic and symptomatic periods was made using a Kruskal- Wallis one-way ANOVA.



**Figure 2:** Bacterial outgrowth. Bacterial titers in CSF, brain (central nervous system compartment), and blood, spleen, and lung (systemic compartment) at the end-stage of disease after inoculation with 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup> and 10<sup>7</sup> CFU *S. pneumoniae* per mouse. Titters are expressed mean CFU/ml +/- S.E.M.

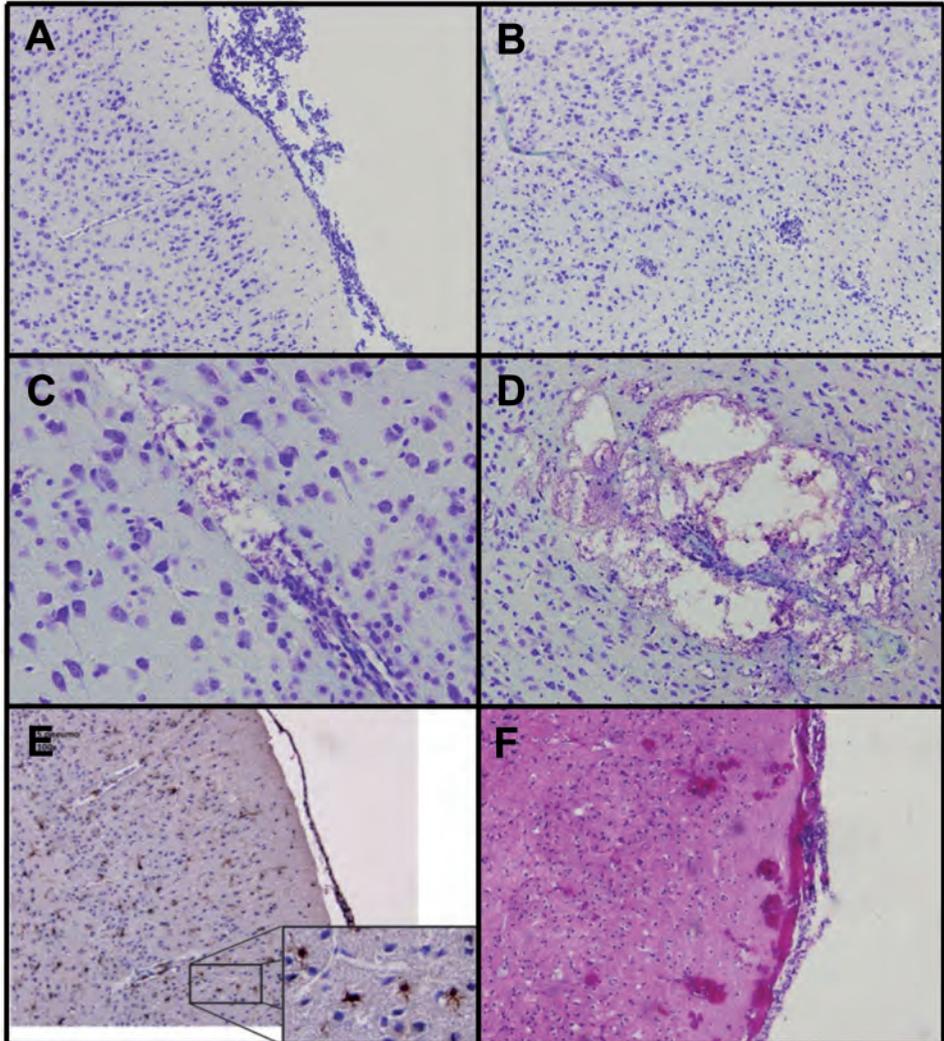
Mice with pneumococcal meningitis showed increased plasma levels of CXCL1 at 6 hours (Figure 3; median 62 versus 213 pg/ml,  $P = 0.004$ ) and 30 hours (median 62 versus 2031 pg/ml,  $P < 0.0001$ ) compared to saline inoculated mice. Furthermore, at 30 hours IL-6 (median 2 versus 202 pg/ml,  $P < 0.001$ ), CXCL2 (median 5 versus 63 pg/ml,  $P = 0.002$ ) and IFN- $\gamma$  (median 3 versus 16 pg/ml,  $P = 0.002$ ) were elevated in plasma of *S. pneumoniae* compared to sham inoculated mice. IL-1 $\beta$ , IL-2, IL-4, IL-10, IL-12p70, IL-17, CCL5, TNF- $\alpha$ , IL-18 and IL-33 were not significantly altered in the plasma of mice with pneumococcal meningitis compared to sham controls.

In brain homogenates, mice with pneumococcal meningitis compared to saline inoculated mice showed elevated levels of CXCL1 and CXCL2 at both 6 hours (Figure 3; CXCL1 median 60 versus 393 pg/ml,  $P < 0.0001$ ; CXCL2 median 45 versus 159 pg/ml,  $P = 0.003$ ) and 30 hours (CXCL1 median 60 versus 18116 pg/ml,  $P < 0.0001$ ; CXCL2 median 45 versus 10637 pg/ml,  $P < 0.0001$ ) time points. IL-6 (median 20 versus 795 pg/ml,  $P < 0.0001$ ), IL-1 $\beta$  (median 165 versus 939 pg/ml,  $P = 0.014$ ), and CCL5 (median 15 versus 1823 pg/ml,  $P < 0.0001$ ) were increased at 30 hours post infection in mice inoculated with pneumococcal meningitis as compared to saline inoculated mice. IL-2, IL-4, IL-10, IL-12p70, IL-17, IFN- $\gamma$ , TNF- $\alpha$ , IL-18 and IL-33 were not altered in brain homogenates



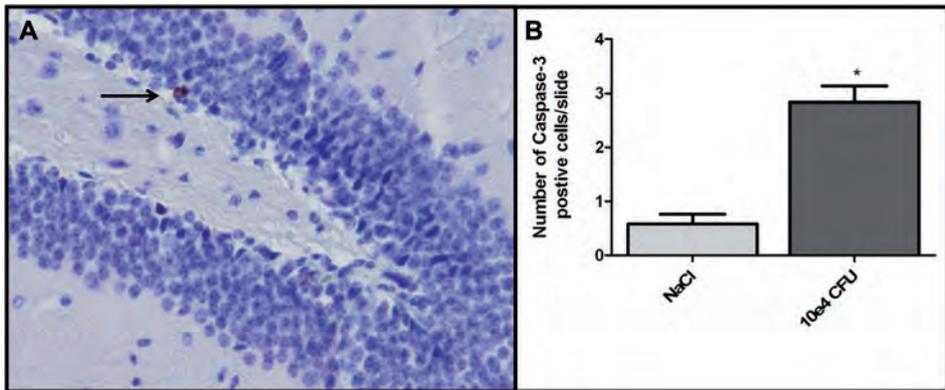
**Figure 3:** Cytokine levels in plasma, brain homogenates and CSF. Median concentrations (expressed in pg/ml) of cytokines in mice inoculated with either NaCl or  $10^4$  CFU/mouse of *S. pneumoniae* and sacrificed after 30 hours, or 6 and 30 hours respectively. Comparisons of cytokine levels between groups were calculated using the Mann-Whitney U test. (\*P < 0.05; \*\* P < 0.01).

of mice with pneumococcal meningitis compared to sham controls. In CSF of mice with pneumococcal meningitis compared to saline inoculated mice, IL-6 (median 26 versus 2772 pg/ml,  $P = < 0.001$ ), CXCL1 (median 84 versus 8369 pg/ml,  $P = 0.002$ ), CXCL2 (median 63 versus 5542 pg/ml,  $P = 0.002$ ) and CCL5 (median 21 versus 309 pg/ml,  $P = 0.005$ ) are elevated 30 hours post infection.



**Figure 4:** Brain pathology of mice with pneumococcal meningitis. Nissl staining of the cortex of mice infected with *S. pneumoniae*, showing extensive leptomenigeal inflammatory infiltrate (A; 100x magnification), perivascular lymphocytic cuffing (B; 100x magnification), perivascular lymphocytic infiltration combined with bacterial overgrowth (C; 200x magnification) and further bacterial overgrowth combined with perivascular necrosis (D; 100x magnification). Iba-1 immunohistochemistry revealed microglial activation (E; 100x magnification). Hematoxylin and eosin staining showed subarachnoidal and cortical hemorrhages (F; 100x magnification).

Histopathology at 6 hours after infection showed high levels of meningeal inflammation in both peripheral and ventricular CSF compartments, but none of the mice had parenchymal lymphocytic infiltration, hemorrhages, microglial activation, or hippocampal apoptosis (Figure 4A). However, 30 hours after inoculation 3 of the 6 mice showed parenchymal lymphocytic infiltration and pockets of bacteria were seen in 2 of 6 mice, located in the perivascular spaces of the penetrating vasculature (Figure 4b). At 30 hours, 5 of 6 mice had one or more parenchymal, mainly cortical, hemorrhages. Three mice demonstrated subarachnoidal hemorrhages. Extensive diffuse microglial activation was observed mice 30 hours after infection and at end stage-stage of disease at all inoculation doses (Figure 4C), although no quantitative analyses were performed. Neuronal apoptosis in the dentate gyrus of the hippocampus was scored independently by two investigators with a kappa of 0.75. A significant increase in hippocampus neuronal apoptosis was observed at 30 hours post infection and was significantly higher than saline infected mice (0.6 vs. 2.8 cells,  $P < 0.001$ ; Figure 5).



**Figure 5:** Neuronal apoptosis. Caspase-3 immunohistochemistry of 10  $\mu$ m sections of the middle portion of the hippocampus of the right cerebral hemisphere of mice inoculated with 10<sup>4</sup> CFU *S. pneumoniae* at 30 hrs post infection (panel A; 200x magnification). Mice inoculated with *S. pneumoniae* showed significantly more hippocampal apoptosis at 30 hrs post infection than NaCl infected control mice (panel B, expressed in mean number of Caspase-3 positive cells/section  $\pm$  S.E.M.; groups were compared using a Student's t-test; \*  $P < 0.001$ ).

## Discussion

We developed a murine model of pneumococcal meningitis in which the histopathological and inflammatory features as well as observed complications resemble clinical and pathological findings in humans following bacterial meningitis.<sup>1,20</sup> The most important features of this model lie in the possibility of combining a relatively low dose of inoculum and long period of disease progression, allowing for a reproducible setting to examine clinical features as well as sufficient time to develop the histopathological features seen in a human setting.

In previous murine models, pneumococcal meningitis was established by either 1) direct bacterial inoculation into the CNS, which generally very short survival times and thus limited use for the study of inflammation processes, or 2) intranasal or intraperitoneal inoculation routes, which more closely models the longer physiological inflammatory mechanisms.<sup>12</sup> Unfortunately, mice inoculated via the intranasal or intraperitoneal route often died as the result of sepsis or pneumonia, and only 50% actually developed meningitis.<sup>19</sup>

In this model the comparison of clinical score progression between mice with different inoculation doses lead to the following conclusions: first, although the pre-symptomatic period was dose dependent (onset of symptoms were later at lower doses of bacterial inoculation), the duration of the symptomatic period was approximately 11.5 hours and similar between groups. This dose-dependent delayed onset provides a model in which direct inoculation in the CNS results in nearly 100% of mice developing meningitis, combined with a prolonged pre-symptomatic period in which various inflammatory mechanisms may be studied. Second, the clinical features contributing to deterioration were largely similar between the 4 different inoculation groups. For example, at the beginning of the symptomatic period (clinical score > 10) the most important contributing factors of clinical deterioration in all four inoculation groups were weight loss, diminished activity and deficits during neurological examination. At the final clinical assessment during the survival experiment, the most important additional factors of clinical deterioration in all 4 groups were the time to turn to upright position and increasing respiratory problems.

TNF- $\alpha$ , IL-1 and IL-6 are considered to be the early response proinflammatory cytokines that are upregulated early in during pneumococcal meningitis.<sup>13</sup> Surprisingly, TNF- $\alpha$  was not elevated at any time-point in our model. Previous animal models demonstrated that TNF- $\alpha$  was mainly increased during the first 6 to 24 hours of the immune response.<sup>20,21</sup> However, human studies show increased CSF levels of TNF- $\alpha$  but only early in the course of the disease.<sup>22,23</sup> This discrepancy may be explained by the lack of measurements that were performed between 6 and 30 hours after infection. IL-1 $\beta$ , which in humans is increased in the first 18 hours of infection, was also markedly increased in brain homogenates, but not in blood in our mice 30 hours after infection.<sup>24</sup> The IL-6 concentrations did significantly increase CSF, brain homogenate and plasma 30 hours after infection. This is consistent with other infection models, in which IL-6, a cytokine displaying both pro- and anti-inflammatory properties, has been shown to be upregulated early during infection. In previous pneumococcal meningitis models IL-6 was shown to be involved in CSF leukocyte recruitment and possibly in the regulation of blood brain barrier disruption.<sup>25,26</sup> The anti-inflammatory cytokine IL-10, which has been shown to downregulate TNF- $\alpha$ , IL-6 and CXCL1 was not measurably increased at any time point.<sup>27</sup>

Of the chemokines, the functional murine IL-8 homologue CXCL1 and CXCL2 were both markedly increased in CSF, brain homogenate and blood at 30 hours after infection. Furthermore, early upregulation of CXCL1 and CXCL2 was also observed as early as 6 hours in brain homogenate, but not in plasma, where only CXCL1 was significantly

increased. In humans, IL-8 has been shown to be elevated in CSF during pneumococcal meningitis, yet in a rabbit meningitis model it was systemic IL-8 that appeared to regulate CSF pleiocytosis.<sup>28,29</sup> CXCL2, which is produced by astrocytes and microglial cells, but also by monocytes and macrophages, has been shown *in vitro* to be a chemoattractant for monocytes and neutrophils recruitment.<sup>29</sup>

Brain histopathology in our model resemble the human situation in pneumococcal meningitis, We found meningeal and parenchymal infiltration, (micro)hemorrhages, perivascular lymphocytic cuffing and perivascular bacterial overgrowth, the beginning of abscess formation, microglial activation, and neuronal apoptosis in the dentate hippocampal gyrus. Parenchymal (micro)hemorrhages were frequently observed (83%) and varied in size and location. These results reflect findings in a recent autopsy series in which microhemorrhages were found in 10 of 16 (67%) patients who died of pneumococcal meningitis.<sup>11</sup> In the clinical setting clinical setting only 1-9% of all patients are documented to have intracranial hemorrhagic complications, which is likely to be an underestimation of the actual number of hemorrhages as only radiological evidence was included.<sup>5</sup> In our model no cortical necrosis was observed at any time point, including in mice that died in the survival studies. Cerebral infarctions occur in approximately 30% of patients with pneumococcal meningitis, and cortical necrosis has been modeled successfully in several rat and infant mouse meningitis models.<sup>6,15,20,30,31</sup> Possible reasons for the absence of necrosis may lie in the duration from inoculation until sacrifice, the choice of animal, age of the mice used, and antibiotic treatments used in other models. The underlying mechanisms for both ischemic stroke and hemorrhages remain unclear, though human CSF studies have suggested dysregulation of local coagulation cascade, complement activation, and diffuse cerebral intravascular coagulopathy.<sup>11,13,32</sup>

The observations of microglial activation at 30 hours after infection reflect *in vitro* findings in which microglial cells are activated after exposure to *S. pneumoniae*.<sup>33</sup> Similarly, the delayed activation of microglial cells supports the results of a previous study in a rabbit model of pneumococcal meningitis in which increased levels of the microglial derived immunomodulatory protein activin A was found at 12 hours after inoculation.<sup>34</sup> Microglia represent a specific subset of cells related to monocytes and dendritic cells and form the initial line of defense of brain parenchyma against damage, injury and infection and become activated in a toll-like receptor dependent fashion upon pneumococcal exposure.<sup>35,36</sup> Upon activation, microglia produce large amounts of proinflammatory cytokines, as well as reactive oxygen and nitrogen intermediates, thereby possibly playing both neuroprotective and neurotoxic roles.<sup>13,37-39</sup> The role of microglia during pneumococcal meningitis is largely unknown at present, but interest has been fueled by the observation that microglial activation *in vitro* is limited by corticosteroids treatment, which has become the standard adjuvant therapy in the treatment of bacterial meningitis in many countries.<sup>2,40,41</sup>

Neuronal apoptosis was first observed in the human autopsy studies of patients who died of bacterial meningitis and was situated in the dentate gyrus of the hippocampus.<sup>9</sup>

Cognitive impairments and more specifically learning difficulties have been attributed to hippocampal apoptosis which has been modeled in mice, rats and rabbits.<sup>15,42,43</sup> Furthermore, the adjuvant treatment of corticosteroids has been suggested as a possible factor aggravating hippocampal apoptosis and reducing learning capacity.<sup>43,44</sup> The process of apoptosis most likely occurs in an early caspase independent and a late caspase dependent mechanism.<sup>45</sup> In this model we were able to detect the late stage caspase-3 dependent apoptosis at 30 hours post infection, providing an additional outcome parameter for further pathophysiological and therapeutic investigations.

## Conclusions

The value of this mouse model is that it provides an experimental setting of pneumococcal meningitis which is highly reproducible, and provides several of the most valuable outcome parameters such as bacterial titers, meningeal and parenchymal infiltration, cytokine profiles, microglial activation, neuronal apoptosis in the hippocampus, perivascular infiltration and (micro) hemorrhages. We feel that the integration of these pathological features, which are characteristic of what is observed in human autopsy studies into a single model, is a valuable tool in the further investigation of both pathophysiological and therapeutic intervention studies.

## Declarations

### Author Contributions

M-K and MG equally participated in the planning and conducting of all the herein mentioned experiments, as well as the writing of the manuscript. DT aided in the histological analyses. TP and DB conceived of the study, participated in design and execution and evaluation of the various experiments. DB provided funding and aided in the drafting of this manuscript. All authors read and approved the final manuscript.

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### Potential conflicts of interest

The authors have no conflicts of interest to report.

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# 4

## Inflammasome activation mediates inflammation and outcome in humans and mice with pneumococcal meningitis

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## Abstract

**Background:** Inflammasomes are multi-protein intracellular signaling complexes that have recently been hypothesized to play a role in the regulation of the inflammation response. We studied associations between inflammasome-associated cytokines IL-1 $\beta$  and IL-18 in cerebrospinal fluid (CSF) of patients with bacterial meningitis and clinical outcome, and pneumococcal serotype. In a murine model of pneumococcal meningitis we examined the pathophysiological roles of two inflammasome proteins, NLRP3 (Nod-like receptor protein-3) and adaptor protein ASC (apoptosis-associated speck-like protein).

**Methods:** In a nationwide prospective cohort study, CSF cytokine levels were measured and related to clinical outcome and pneumococcal serotype. In a murine model of pneumococcal meningitis using *Streptococcus pneumoniae* serotype 3, we examined bacterial titers, cytokine profiles and brain histology at 6 and 30 hours after inoculation in wild-type (WT), *Asc* and *Nlrp3* deficient mice.

**Results:** In patients with bacterial meningitis, CSF levels of inflammasome associated cytokines IL-1 $\beta$  and IL-18 were related to complications, and unfavorable disease outcome. CSF levels of IL-1 $\beta$  were associated with pneumococcal serotype ( $p < 0.001$ ). In our animal model, *Asc* and *Nlrp3* deficient mice had decreased systemic inflammatory responses and bacterial outgrowth as compared to WT mice. Differences between *Asc*<sup>-/-</sup> and WT mice appeared sooner after bacterial inoculation and were more widespread (lower pro-inflammatory cytokine levels in both blood and brain homogenate) than in *Nlrp3*<sup>-/-</sup> mice. *Nlrp3* deficiency was associated with an increase of cerebral neutrophil infiltration and cerebral hemorrhages when compared to WT controls.

**Conclusions:** Our results implicate an important role for inflammasome proteins NLRP3 and ASC in the regulation of the systemic inflammatory response and the development of cerebral damage during pneumococcal meningitis, which may dependent on the pneumococcal serotype.

## Introduction

Bacterial meningitis is a life threatening infectious disease of the central nervous system that affects between 2.6 and 6.0 people per 100 000 per year in Europe and may be up to ten times higher in developing countries. The most common causative organism of community acquired bacterial meningitis in adults is *Streptococcus pneumoniae*, which is responsible for two-thirds of cases in Europe and United States.<sup>1</sup> Pneumococcal meningitis has a case fatality rate of 16%-37% and of the survivors 30-52% suffer from neurological sequelae.<sup>2,3</sup> There remains a need for better (adjuvant) therapies, for which further understanding of underlying pathophysiology is necessary.<sup>4</sup>

Recently, several studies have examined the role of inflammasomes in bacterial meningitis. Inflammasomes are intracellular multiprotein complexes, belonging to the family of Nod-like receptors (NLRs), and are triggered by exposure to microbial and endogenous danger signals such as ATP, changes in K<sup>+</sup> concentration, oxygen radicals and uric acid released through cell injury in inflammation.<sup>5-7</sup> Upon activation, NLRP3 binds to pro-caspase via adaptor apoptosis-associated speck-like protein (ASC), which is shared by several inflammasome types. Pro-caspase-1 is converted to activated caspase-1, which subsequently converts interleukins 1beta (IL-1 $\beta$ ) and IL-18 into their active secreted forms.<sup>5-7</sup> Recently however, caspase-independent proinflammatory activity of NLRP3 has also been described.<sup>8,9</sup> To date, four inflammasomes have been characterized, of which NLRP3 has been the most extensively researched.

Further examination of the role of inflammasomes in pneumococcal meningitis is of interest for several reasons: First, inflammasomes are the well established activators of caspase-1, which has been shown to be elevated in the cerebral spinal fluid (CSF) of patients with pneumococcal meningitis compared to non-infected controls.<sup>10,11</sup> Moreover, mice deficient of caspase-1 displayed less severe inflammation, decreased brain water content and improved clinical score in a pneumococcal meningitis model.<sup>10,12</sup> Second, IL-1 $\beta$ , which is activated by caspase-1, has been shown to be elevated in the CSF of children with pneumococcal meningitis and correlates with disease severity, a finding that also has been observed in various animal models.<sup>11,13-15</sup> Lastly, several murine models have demonstrated the importance of NLRP3 in the pathophysiology of invasive pneumococcal disease.<sup>16,17</sup> Most notably, a recent study showed that NLRP3 mediates brain damage in an experimental meningitis model using a serotype 2 *S. pneumoniae*.<sup>18</sup>

In this study we measured the CSF levels of inflammasome related cytokines IL-1 $\beta$  and IL-18 in a prospective nationwide cohort of community acquired bacterial meningitis and correlated these to clinical data and pneumococcal serotype. We then investigated the role of inflammasome gene NLRP3 and adapter protein ASC in a murine model of meningitis using serotype 3 *S. pneumoniae*, a common serotype in pneumococcal meningitis.<sup>19</sup>

## Methods

### Patients cohort

In a nationwide prospective cohort study we included bacterial meningitis patients older than 16 years of age with positive CSF cultures who were identified by The Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) from March 2006 to June 2009. The NRLBM provided the names of the hospitals where patients with bacterial meningitis had been admitted 2–6 days previously. The treating physician was contacted, and informed consent was obtained from all participating patients or their legally authorized representatives. Outcome was graded at discharge according to the Glasgow Outcome Scale, a well-validated instrument with good interobserver agreement.<sup>20</sup> A score of one on this scale indicates death; a score of two a vegetative state; a score of three severe disability; a score of four moderate disability; and a score of five mild or no disability. A favorable outcome was defined as a score of five, and unfavorable outcome as a score of one to four. The study was approved by the medical ethical (review) committee of the Academic Medical Center of Amsterdam.

### IL-1 $\beta$ and IL-18 measurements in CSF samples of patients with bacterial meningitis

We measured IL-1 $\beta$  and IL-18 in the CSF of 289 patients with bacterial meningitis included in the cohort and 19 controls with luminex<sup>®</sup> technology using a Milliplex assay (Millipore, Billerica, MA, USA). CSF from the first diagnostic tap was collected, centrifuged and supernatant was aliquoted and stored at  $-80^{\circ}\text{C}$  until analysis. Controls were patients evaluated for acute headache, without signs of meningitis and normal CSF findings. In these patients a subarachnoid hemorrhage was excluded as cause of their headache by CSF examination. Leftover CSF was collected, centrifuged and supernatant was stored at  $-80^{\circ}\text{C}$  until analysis.

### Mouse model and tissue preparation

A well characterized and previously described murine model of pneumococcal meningitis was used in this study.<sup>21</sup> *Nlrp3*<sup>-/-</sup> mice with C57BL/6 background (kind gift of Richard Flavell, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT, USA) and *Asc*<sup>-/-</sup> mice with C57BL/6 background (kind gift of Fayyaz Sutterwala, University of Iowa, Iowa City, IA, USA), and specific pathogen-free C57BL/6 mice (Charles River, Wilmington, MN, USA) were weighed, clinically examined, and scored clinically. Inoculations were performed in several rounds, all with male mice aged 8–12 weeks. In each inoculation round knockout mice and an equal number of wild-type mice were inoculated with the same bacterial inoculum to control for variations between inocula. Experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, The Netherlands.

Serotype 3 *S. pneumoniae* (ATCC 6303; American Type Culture Collection, Rockville, MD, USA) was grown to mid log phase in 4 hours at  $37^{\circ}\text{C}$  in Todd-Hewitt

broth supplemented with yeast (Difco, Detroit, MI). Pneumococci were harvested by centrifugation at 4000 rpm for 10 min, and washed twice with sterile isotonic saline. Bacteria were diluted to a final concentration of  $1 \times 10^6$  CFU/ml and serial ten-fold dilutions were plated on sheep blood agar plates for quantification.

Mice were inoculated in the cisterna magna under isoflurane anesthesia with 10  $\mu$ l saline containing  $1 \times 10^4$  CFU (range  $0.6 \times 10^4 - 1.2 \times 10^4$  CFU) of *S. pneumoniae* or sterile saline alone. Twelve mice per group (WT, *Nlrp3*<sup>-/-</sup> and *Asc*<sup>-/-</sup>) were inoculated with *S. pneumoniae* and six mice per group (WT, *Nlrp3*<sup>-/-</sup> and *Asc*<sup>-/-</sup>) with sterile saline. After intracisternal inoculation mice were assessed for neurologic damage as a result of the puncture, which was not present in any of the mice. At 6 or 30 hours post infection mice were anesthetized by intraperitoneal injection of ketamine (Eurovet Animal Health, Bladel, the Netherlands) and medetomidine (Pfizer Animal Health, Capelle aan den IJssel, the Netherlands) followed by cardiac puncture for blood collection and perfusion of organs with sterile isotonic saline via the left ventricle.

CSF was collected by puncture of the cisterna magna, and brains, lungs and spleen were harvested. The right hemisphere was suspended in 10% buffered formalin and embedded in paraffin for histopathology. The left hemisphere and spleen were taken up in 20% weight per volume sterile saline and were disrupted with a tissue homogenizer. Serial ten-fold dilutions of blood, CSF, brain homogenate and spleen homogenate were plated on sheep-blood agar plates and bacteria were allowed to grow overnight at 37°C. Heparin blood was centrifuged at 4000 rpm for 5 min. at 4°C. Tissue homogenates were lysed by adding 1:1 two times concentrated lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM MgCl(H<sub>2</sub>O)<sub>6</sub>, 1 mM CaCl<sub>2</sub>(H<sub>2</sub>O)<sub>2</sub>, 1% Triton, AEBSF 4  $\mu$ g/ml, EDTA-NA2 50  $\mu$ g/ml, pepstatin 10 ng/ml, leupeptin 10 ng/ml, pH 7.4), incubating on ice for 30 min. and centrifuged at 4000 rpm for 5 min. at 4°C. Plasma and lysed tissue supernatant were removed and stored at -20°C for further analysis.

#### RT-PCR

Total RNA was isolated from murine brain homogenates with the Nucleospin® RNA II Purification kit (Clontech Laboratories, Mountain View, CA, USA; Bioke, Leiden, the Netherlands). Isolated RNA was converted to cDNA using oligo(dT) primer (Promega, Leiden, the Netherlands), Moloney murine leukemia virus reverse transcriptase (Invitrogen, Breda, the Netherlands), RT-buffer (Promega, Leiden, the Netherlands), deoxynucleotide triphosphate mix (Invitrogen, Breda, the Netherlands), dithiothreitol (Duchefa Farma, Haarlem, the Netherlands) and RNase inhibitor (Invitrogen, Breda, the Netherlands). After incubation for 10 min at 23°C, RT was carried out for 60 min at 42°C, followed by RT inactivation for 3 min at 95°C. Reverse transcription-PCR's (RT-PCR's) were performed with LightCycler SYBR green I master mix (Roche, Mijdrecht, the Netherlands) and measured in a LightCycler 480 (Roche) apparatus under the following conditions: 5 min 95°C hot start, followed by 40 cycles of amplification (95°C for 15 sec - 60°C for 5 sec - 72°C for 20 sec). For quantification, standard curves were constructed

on serial dilutions of a sample with known high cDNA content. Data were analyzed using LightCycler software. Gene expression is presented as a ratio of the expression of the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used for RT-PCR were as follows: mouse GAPDH, 5'-CTCATGACCACAGTCCATGC-3' (forw) and 5'-CACATTGGGGGTAGGAACAC-3' (rev); mouse *Nlrp3*, 5'-CCACAGTGTAACCTGCAGAAGC-3' (forw) and 5'-GGTGTGTGAAGTTCGGTTGG-3' (rev); mouse *Asc*, 5'-GGTGTGTGAAGTTCGGTTGG-3' (forw) and 5'-GGTGTGTGAAGTTCGGTTGG-3' (rev).

#### Cytokine measurements in murine tissue

IL-1 $\beta$ , IL-6, KC/CXCL1, TNF- $\alpha$ , IL-18, and MIP-2/CXCL2 were measured in plasma and brain homogenates with luminex® technology using a mouse cytokine and chemokine Bioplex kit (Bio-Rad Laboratories, Veenendaal, The Netherlands). Luminex assays were analysed on a Luminex 200 with Bio-Plex Manager software 5.0. Samples were 4 times diluted. Mouse myeloperoxidase (MPO) was measured by ELISA (Hycult Biotechnology, Uden, The Netherlands). Mouse albumin was measured by ELISA (GenWay Biotech, San Diego, CA).

#### Murine histopathology

Five  $\mu\text{m}$  paraffin brain sections were cut in a coronal plane from the olfactory bulb to the beginning of the cerebellum, and sections at intervals of 1400  $\mu\text{m}$  or intervals of 700  $\mu\text{m}$  throughout the hippocampal region were selected. Sections were mounted on slides and stained with hematoxylin and eosin. To assess differences in brain damage, coronal cut brain sections of WT and *Asc*<sup>-/-</sup> and *Nlrp3*<sup>-/-</sup> mice were scored for intracerebral hemorrhages, subpial hemorrhages, cerebral infarctions, and for neutrophil influx on a five point scale: 0) normal histopathology; 1) few inflammatory cells in the meninges but no perivascular cuffing; 2) moderate number of inflammatory cells in the meningitis and cuffing of some of the vessels; 3) extensive number of inflammatory cells in the meninges, prominent perivascular cuffs with mild infiltration of the neutrophil; 4) extensive number of inflammatory cells in the meninges, prominent perivascular cuffs, the presence of many inflammatory cells in the neutrophil and intraparenchymal pocket formation. Sections of mice 30 hours after induction of pneumococcal meningitis (n=8 per group) were scored. Sections were scored by two independent observers blinded for the experimental groups (interobserver kappa 0.75).

#### Statistics

The Mann-Whitney *U* test was used to identify differences in baseline characteristics, bacterial outgrowth, cytokine levels and histopathological scores among groups with respect to continuous variables. Dichotomous variables were compared using the  $\chi^2$  test. Correlation analyses were performed with the Spearman's rank correlation coefficient. For all analyses a P-value < 0.05 was considered significant.

## Results

### CSF IL-1 $\beta$ and IL-18 levels in patients with bacterial meningitis are associated with complications and unfavorable disease outcome.

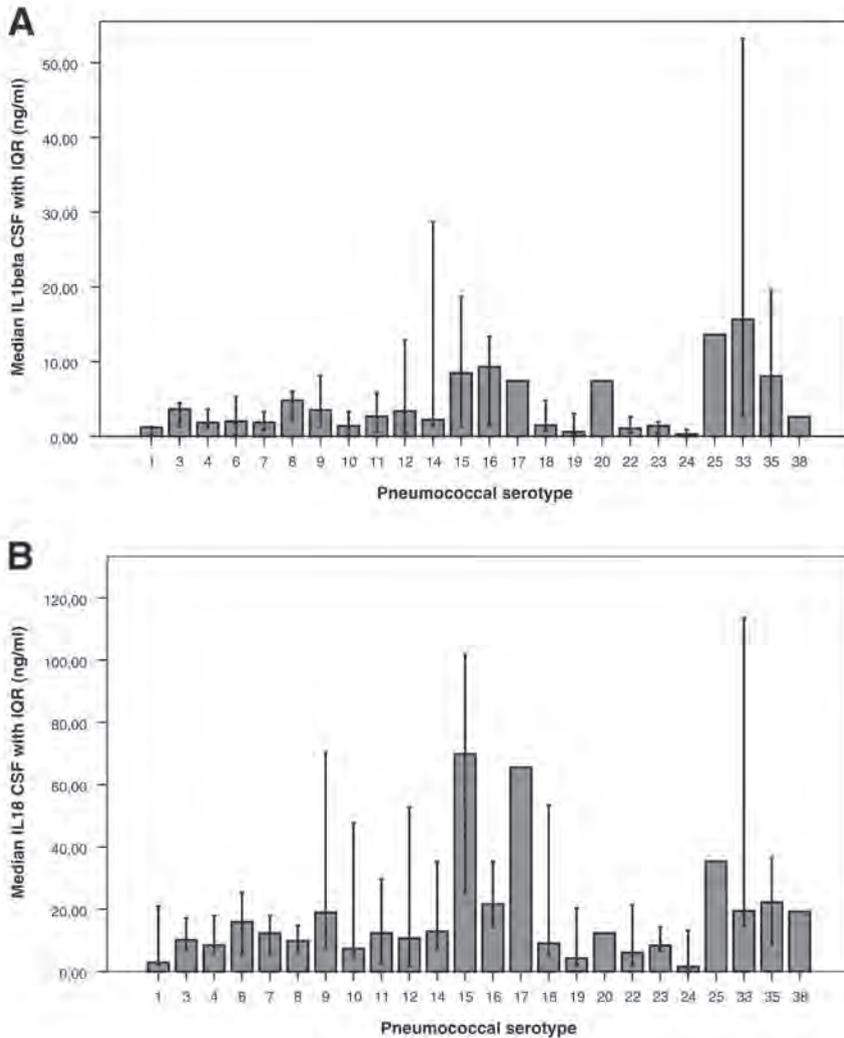
A total of 801 Dutch patients with bacterial meningitis were included as described previously.<sup>22</sup> In this study the distribution of causative organisms was: 576 episodes (72%) *S. pneumoniae*, 92 (12%) *Neisseria meningitidis*, 41 (5%), *Listeria monocytogenes*, and other bacteria in 92 (12%) episodes. The case fatality rate was 18%, and 38% of patients had poor clinical functional outcome as defined as a score of 1–4 on the Glasgow Outcome Scale. CSF was available in 289 of the episodes with bacterial meningitis (36%), and 211 of 576 with pneumococcal meningitis (35%). Levels of IL-1 $\beta$  and IL-18 were elevated in the CSF of patients with meningitis as compared to controls. Higher IL-1 $\beta$  levels were associated with occurrence of systemic complications (median 0.91 ng/ml [IQR 0.15-3.00] versus 2.02 ng/ml [IQR 0.33-5.26],  $p=0.001$ ) and neurologic complications (median 0.81 ng/ml [IQR 0.15-3.36] versus 1.60 ng/ml [IQR 0.29-4.73],  $p=0.020$ ). IL-1 $\beta$  levels were higher in patients with an unfavorable outcome although this difference was not statistically significant (median 1.04 ng/ml [IQR 0.17-3.65] versus 1.53 ng/ml [IQR 0.27-5.16],  $p=0.11$ ). High IL-18 levels were also associated with systemic complications (median 8.50 ng/ml [IQR 3.07-20.71] versus 15.13 ng/ml [IQR 6.35-27.32],  $p=0.006$ ) and unfavorable outcome (median 9.27 ng/ml [IQR 3.36-22.83] versus 14.65 ng/ml [IQR 5.53-26.97],  $p=0.037$ ). In the subgroup of patients with pneumococcal meningitis ( $n=211$ ) associations with systemic complications remained significant.

### CSF IL-1 $\beta$ and IL-18 levels in patients with bacterial meningitis are associated with pneumococcal serotype.

Pneumococcal serotyping was performed in 509 pneumococcal strains (88%) and the most common serotypes were 3, 23 and 7 (Table III; serotype distribution has been partly published previously).<sup>23</sup> CSF levels of IL-1 $\beta$  were related to pneumococcal serotype (Kruskal-Wallis 1 way ANOVA,  $p<0.001$ ; Figure 1).

### ASC and NLRP3 expression in *Asc* and *Nlrp3* knockout mice.

To confirm that the inflammasome components ASC and NLRP3 were expressed or knocked out in our meningitis mouse model, we examined mouse brain homogenates from WT mice infected with *S. pneumoniae* serotype 3. At 6 and 30 h after infection ASC and NLRP3 expression in brain homogenates appeared upregulated as compared to saline inoculated mice (Figure 2). *Nlrp3*<sup>-/-</sup> mice showed expression of ASC and no NLRP3, and conversely *Asc*<sup>-/-</sup> mice showed expression of NLRP3 and no ASC.

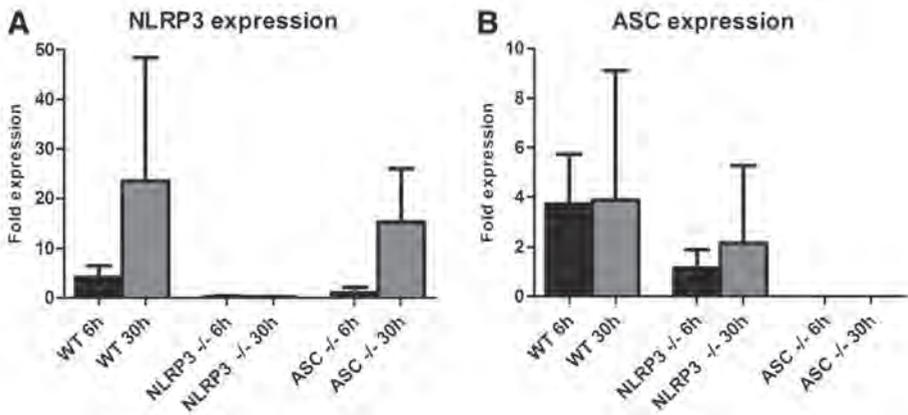


**Figure 1:** Median levels of IL-1 $\beta$  (A) and IL-18 (B) with interquartile range by pneumococcal serotype. CSF levels of IL-1 $\beta$  and IL18 were related to pneumococcal serotype (Kruskal-Wallis 1 way ANOVA,  $p < 0.001$ ).

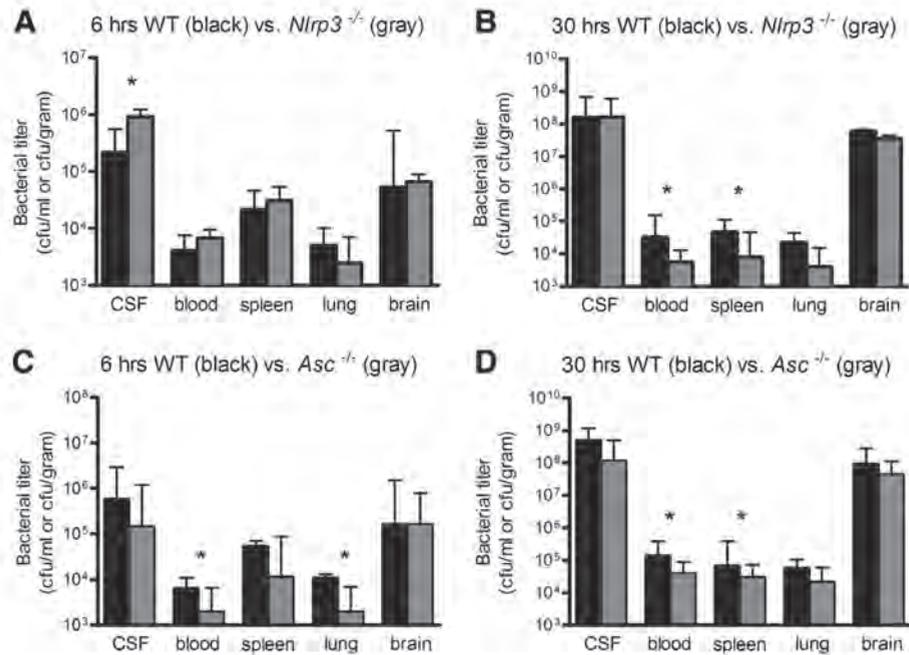
### Decreased systemic bacterial loads in *Asc* and *Nlrp3* knockout mice.

*Nlrp3*<sup>-/-</sup> mice showed more bacterial outgrowth in CSF at 6 h compared to WT mice (median  $9.2 \times 10^5$  CFU/ml versus  $2.2 \times 10^5$  CFU/ml,  $p = 0.046$  Figure 3A). However, *Nlrp3*<sup>-/-</sup> had less bacterial outgrowth in blood and spleen at 30 h, as compared to WT mice (blood,  $5.7 \times 10^3$  CFU/ml versus  $3.2 \times 10^4$  CFU/ml,  $p = 0.017$ ; spleen,  $8.0 \times 10^3$  CFU/ml versus  $4.7 \times 10^5$  CFU/ml,  $p = 0.012$  Figure 3B). No differences in bacterial outgrowth in brain homogenates were observed. *Asc*<sup>-/-</sup> mice showed less bacterial outgrowth at 6 h in blood and lung compared to WT mice (blood,  $2.0 \times 10^3$  CFU/ml versus  $6.6 \times 10^3$  CFU/ml,  $p = 0.017$ ; lung,  $2.0 \times 10^3$  CFU/ml versus  $1.1 \times 10^5$  CFU/ml,  $p = 0.043$  Figure 3C). At 30 h *Asc*<sup>-/-</sup> mice showed less bacterial outgrowth in blood ( $3.9 \times 10^4$  CFU/ml versus  $1.4 \times 10^5$  CFU/ml,

p=0.039 Figure 3D) and spleen ( $3.1 \times 10^4$  CFU/ml versus  $6.7 \times 10^4$  CFU/ml, p=0.016). No differences in bacterial outgrowth in CSF or brain homogenates in *Asc*<sup>-/-</sup> mice were observed.



**Figure 2:** Expression of NLRP3 and ASC in brain homogenates. Expression of NLRP3/GAPDH (A) and ASC/GAPDH (B) in brain homogenates of WT, *Asc*<sup>-/-</sup> and *Nlrp3*<sup>-/-</sup> mice 6 hours and 30 hours after induction of *S. pneumoniae* meningitis compared to mice inoculated with saline. Three mice per group were analysed in the sham infected mice and the 6 hour timepoint; 4 mice were analyzed at the 30 hour timepoint. Data are given as means  $\pm$  SD.

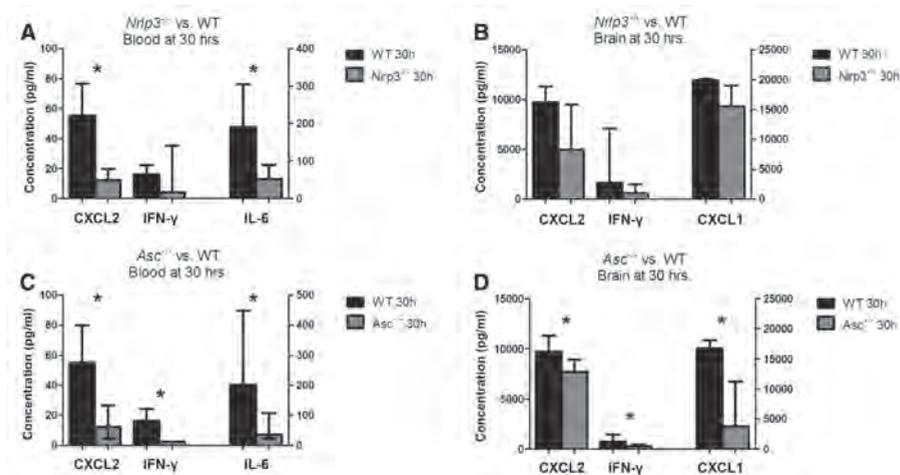


**Figure 3:** Bacterial outgrowth in blood, spleen, lung brain and CSF. Bacterial titers in WT vs., *Nlrp3*<sup>-/-</sup> mice at 6 (A) and 30 hours (B); and WT vs. and *Asc*<sup>-/-</sup> mice at 6 (C) and 30 hours (D) after induction of pneumococcal meningitis. Twelve mice per group were analyzed. Data are given as medians and 75th quartile. \* P < 0.05.

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### Decreased systemic inflammatory response in both *Nlrp3* and *Asc* knockout mice at 6 and 30 hrs.

*Nlrp3*<sup>-/-</sup> mice showed decreased plasma levels of CXCL2 (median 12 pg/ml [IQR 5–20] versus 55 pg/ml [IQR 5–77], *p*=0.037) and IL-6 (52 pg/ml [IQR 24–90] versus 191 pg/ml [70–306], *p*=0.019) at 30 h as compared to WT mice (Figure 4A). No significant cytokine differences were found at 6 hours. *Asc*<sup>-/-</sup> mice showed decreased plasma levels of CXCL2 (12 pg/ml [IQR 5–27] versus 55 pg/ml [IQR 35–80] pg/ml, *p*=0.01), IL-6 (37 pg/ml [IQR 24–108] versus 202 pg/ml [IQR 46–448], *p*=0.034) and IFN- $\gamma$  (3 pg/ml [3] versus 16 pg/ml [3–24], *p*=0.005) at 30 h, as compared to WT mice. At 6 h only CXCL1 levels were lower (105 pg/ml [78–202] versus 186 pg/ml [151–388], *p*=0.005). Notably, plasma levels of IL-1 $\beta$  and IL-18 were similar in *Nlrp3*<sup>-/-</sup>, *Asc*<sup>-/-</sup> and WT mice (Figure 4C). For the other measured cytokines no significant difference was found at 6 or 30 hours.



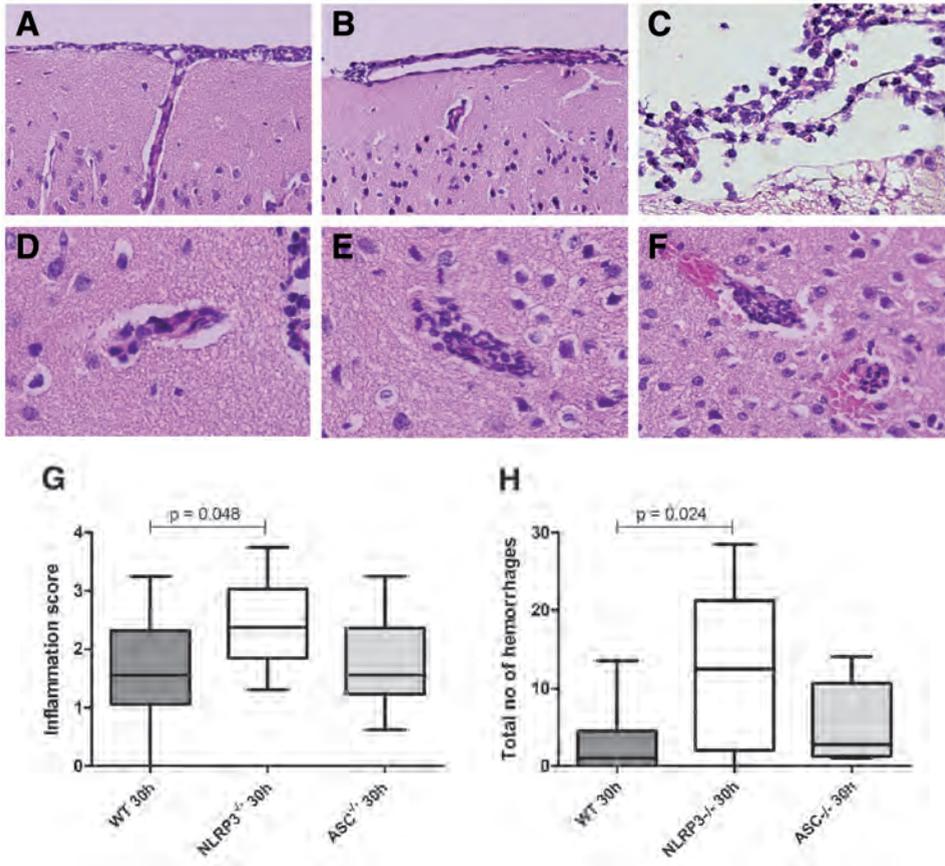
**Figure 4:** Cytokine levels in blood and brain homogenate. Cytokines were measured in WT (*n* = 11), *Nlrp3*<sup>-/-</sup> (*n* = 12) and *Asc*<sup>-/-</sup> (*n* = 12) mice in blood (A, C) and brain homogenate (WT; *n* = 7, *Nlrp3*<sup>-/-</sup>; *n* = 8, *Asc*<sup>-/-</sup>; *n* = 8) (B, D) at 30 hours after induction of pneumococcal meningitis. Undisplayed cytokines measurement did not differ significantly between *Nlrp3*<sup>-/-</sup> or *Asc*<sup>-/-</sup> and WT mice. Data are given as medians and 75th quartile. \* *P* < 0.05.

### Decreased brain cytokine and chemokine levels in *Asc*<sup>-/-</sup> but not *Nlrp3*<sup>-/-</sup> mice.

*Nlrp3*<sup>-/-</sup> mice showed no differences in cytokine responses in the brain compared to WT mice, and brain albumin levels were also similar between WT and *Nlrp3*<sup>-/-</sup> mice (data not shown). *Asc*<sup>-/-</sup> mice displayed lower levels of CXCL1 (median, 3.81 ng/ml [IQR 1.57–11.24] versus 16.75 ng/ml [IQR 11.49–18.12], *p*=0.040), CXCL2 (7.74 ng/ml [IQR 1.46–8.91] vs. 9.74 ng/ml [IQR 8.20–11.3], *p*=0.049) and IL-6 (0.30 ng/ml [IQR 0.10–0.45] versus 0.76 ng/ml [IQR 0.61–1.45], *p*=0.049) than WT mice in brain homogenates at 30 h (Figure 4D). Consistent with this finding, brain albumin concentrations were decreased in *Asc*<sup>-/-</sup> mice compared to WT mice at 30 h (*p*=0.026), indicating attenuated blood brain barrier disruption in *Asc*<sup>-/-</sup> mice compared to WT mice. No differences in brain IL-1 $\beta$  or IL-18 levels were measured between WT and *Nlrp3*<sup>-/-</sup> or *Asc*<sup>-/-</sup> mice.

### Enhanced brain damage in *Nlrp3*<sup>-/-</sup> but not in *Asc*<sup>-/-</sup> mice.

Neutrophil infiltrate in the brain was more pronounced in *Nlrp3*<sup>-/-</sup> mice at 30 h after inoculation as compared to WT mice (median score 1.5 versus 2.4,  $p=0.018$ ; Figure 5G). *Nlrp3*<sup>-/-</sup> mice also showed an elevated number of intracerebral and subpial hemorrhages and as compared to WT mice (median 12.5 versus 1.0 per slide,  $p=0.02$ ; Figure 5H). *Asc*<sup>-/-</sup> mice showed no difference in neutrophil influx score and intracerebral hemorrhages compared to WT mice. Brain MPO levels were similar in both knockouts and wild-type mice (data not shown).



**Figure 5:** Histopathology in *Asc*<sup>-/-</sup>, *Nlrp3*<sup>-/-</sup>, and WT mice with pneumococcal meningitis. Representative brain slides showing neutrophil infiltration in the meninges 30 hours after induction of pneumococcal meningitis in a WT mouse (A), *Asc*<sup>-/-</sup> mouse (B) and *Nlrp3*<sup>-/-</sup> mouse (C). Perivascular cuffing 30 hours after induction of pneumococcal meningitis in a WT mouse (D), *Asc*<sup>-/-</sup> mouse (E) and *Nlrp3*<sup>-/-</sup> mouse (F), showing frequent intracerebral and subpial hemorrhages associated with neutrophil infiltration; meningeal, perivascular and intracerebral neutrophil influx was scored on a scale 0–4, means of 16 brain slides per mouse in the coronal plane are displayed (WT,  $n=11$ ; *Asc*<sup>-/-</sup>,  $n=8$ ; *Nlrp3*<sup>-/-</sup>,  $n=9$ ; G). Sum of intracerebral hemorrhages and subpial hemorrhages per mouse (WT,  $n=11$ ; *Asc*<sup>-/-</sup>,  $n=8$ ; *Nlrp3*<sup>-/-</sup>,  $n=9$ ; H).

## Discussion

This study implicates an important role for inflammasomes in regulation of systemic inflammation and development of cerebral damage during pneumococcal meningitis. In our patient cohort, inflammasome associated cytokines IL-1 $\beta$  and IL-18 levels in CSF of patients with bacterial meningitis correlated with development of systemic complications and unfavorable prognosis; and in the subgroup of patients with pneumococcal meningitis, IL-1 $\beta$  and IL-18 correlated with systemic complications only. In our murine model of pneumococcal meningitis, deficiency of inflammasome components ASC and NLRP3 led to decreased systemic inflammatory responses and bacterial outgrowth in the systemic compartment as compared with WT mice. Conversely, *Nlrp3* deficiency led to enhanced central nervous system inflammation and increased brain damage. Differences between *Asc*<sup>-/-</sup> and WT mice occurred sooner after intrathecal inoculation with *S. pneumoniae* (lower bacterial titers and CXCL1 serum levels at 6 h) and were more widespread (lower pro-inflammatory cytokine levels in both the systemic compartment (blood) and central nervous system compartment (brain homogenate)) than in the *Nlrp3*<sup>-/-</sup> mice.

In our murine model, NLRP3 was protective for brain damage, as *Nlrp3*<sup>-/-</sup> mice had enhanced cerebral neutrophil influx and an increased number of cerebral hemorrhages. NLRP3 has been investigated with regard to pneumococcal infections in both lung infection models and a meningitis model, but findings are not unanimous. In a lung-infection model, *Nlrp3*<sup>-/-</sup> mice have higher bacterial titers and a higher mortality than WT controls.<sup>16,18,24</sup> In a murine model of pneumococcal meningitis, better clinical outcome and decreased brain inflammation in *Nlrp3*<sup>-/-</sup> (and *Asc*<sup>-/-</sup>) mice was found as compared to WT controls.<sup>18</sup> Blocking of IL-1 $\beta$  and IL-18 in this meningitis model, led to a decrease in disease severity and which prompted the suggestion that the NLRP and ASC dependent changes are solely IL-1 and IL-18 related.<sup>18</sup> Our findings that IL-1 $\beta$  and IL-18 levels were not significantly altered in *Nlrp3*<sup>-/-</sup> or *Asc*<sup>-/-</sup> mice, must be interpreted with caution as no assays are available that can discriminate between the pro- and active forms of murine IL-1 $\beta$  and IL-18. Previous studies showed increased IL-1 $\beta$  levels in brain homogenates of WT mice with pneumococcal meningitis at 30 hours compared to sham.<sup>21</sup> We did not perform experiments blocking IL-1 $\beta$ , IL-18 or Caspase-1 in our model to further elucidate this mechanism.

Discrepancies in brain damage in *Nlrp3*<sup>-/-</sup> mice between our study and the previous study in experimental pneumococcal meningitis may be explained by the different pneumococcal serotypes used to establish meningitis between both models.<sup>18</sup> We inoculated mice with a *S. pneumoniae* serotype 3 strain as opposed to the serotype 2 strain used in the previous study. Furthermore, we used a lower intrathecal dose of *S. pneumoniae* than the previous study (10<sup>4</sup> CFU vs. 10<sup>5</sup>/10<sup>6</sup> CFU). *S. pneumoniae* serotype 2 is less heavily encapsulated and less virulent than serotype 3 and needs high doses to induce infection.<sup>25</sup> An *in vitro* study showed that high bacterial loads of *S. pneumoniae* serotype 2 are needed before IL-1 $\beta$  concentration in cell culture supernatants are elevated.<sup>26</sup>

The variation of immune response between different serotypes of *S. pneumoniae* has been demonstrated by several groups.<sup>25,27</sup> In our patient cohort, we observed that the most common pneumococcal serotypes were 3, 23, and 7, and that CSF levels of IL-1 $\beta$  was serotype related. This observation may be due to serotype specific properties of the pneumococcal capsule. Alternatively, pneumolysin, a pore-forming toxin which is known to interact directly with the innate immune system (through, for instance complement or binding of Toll Like Receptor-4), is secreted in varying amounts depending on bacterial serotype. Pneumolysin has been reported to have both inflammasome inhibiting and activating properties, which may be caused by the recently described effects of pneumolysin polymorphisms on innate immune system recognition.<sup>16,17,28</sup> We chose *S. pneumoniae* serotype 3 for our animal studies, as it is one of the most commonly encountered serotypes among patients with pneumococcal meningitis.<sup>19</sup>

The more pronounced phenotype of the *Asc*<sup>-/-</sup> mice as compared to the *Nlrp3*<sup>-/-</sup> mice with pneumococcal meningitis can be explained by other, NLRP3 inflammasome independent, functions of ASC. The (functional) relationship between ASC, NLRP3, and caspase-1 activation during pneumococcal infection was recently described in murine pneumonia model, in which *S. pneumoniae* infection led to caspase-1 activation and IL-1 $\beta$ /IL-18 maturation through the activation of both the NLRP3 and the AIM2 (absent in melanoma) inflammasomes, in a process which was completely absent in the ASC deficient mice. Furthermore, ASC is capable of binding and facilitating the function of several other inflammasomes (such as NLRC4 and IFI16), though the relevance of this during pneumococcal infection is not evident.<sup>24,29</sup> Lastly, independently of the inflammasomes, ASC has been shown to potent regulator of a large number of inflammatory and cell-death related genes.<sup>30</sup> The observation that NLRP3 deficient mice but not ASC deficient mice, expressed more brain damage suggests a protective mechanism in which NLRP3 may act independently of ASC and of the NLRP3 inflammasome. Such “inflammasome-independent” role of NLRP3 in tissue injury has been described in a mouse model of renal ischemia-reperfusion injury, though a mechanism remains unclear.<sup>8,9</sup>

## Conclusion

In conclusion, although a definite mechanism remains elusive, our results provide additional evidence for an important role of inflammasomes (specifically the NLRP3 and ASC proteins and inflammasome associated cytokines IL-1 $\beta$  and IL-18) in the regulation of an inflammatory response and brain damage during pneumococcal meningitis. Further human and animal studies are necessary to clarify the pathophysiological mechanism, as well as explore the possibility of interference of inflammasome activation as a potential adjunctive therapy in the treatment of pneumococcal meningitis.

## Declarations

### Author Contributions

MG and BM-K participated equally in the planning and conducting of all the herein mentioned experiments, as well as the writing of the manuscript. MB aided in the data analysis as well as drafting of this manuscript. DT and JL aided in the histological analyses. RF provided the transgenic mice needed for the murine studies. AE aided in the processing and analysis of cerebral spinal fluid. TP and DB conceived of the study, participated in design and execution and evaluation of the various experiments. DB provided funding and aided in the drafting of this manuscript. All authors read and approved the final manuscript.

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### Potential conflicts of interest

The authors have no conflicts of interest to report.

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# 5

## Genetic variation in inflammasome genes is associated with outcome in bacterial meningitis

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## Abstract

Bacterial meningitis is a severe and deadly disease, most commonly caused by *Streptococcus pneumoniae*. Disease outcome has been related to severity of the inflammatory response in the subarachnoid space. Inflammasomes are intracellular signaling complexes contributing to this inflammatory response. The role of genetic variation in inflammasome genes in bacterial meningitis is largely unknown. In a prospective nationwide cohort of patients with pneumococcal meningitis, we performed a genetic association study and found that single-nucleotide polymorphisms in the inflammasome genes *CARD8* (rs2043211) and *NLRP1* (rs11621270) are associated with poor disease outcome. Levels of the inflammasome associated cytokines interleukin (IL)-1 $\beta$  and IL-18 in cerebrospinal fluid also correlated with clinical outcome, but were not associated with the *CARD8* and *NLRP1* polymorphisms. Our results implicate an important role of genetic variation in inflammasome genes in the regulation of inflammatory response and clinical outcome in patients with bacterial meningitis.

## Introduction

Bacterial meningitis is associated with high mortality, even in developed countries despite the implementation of childhood vaccination programs and effective antimicrobial agents.<sup>1,2</sup> The most common causative agent is *Streptococcus pneumoniae*, with case fatality rates ranging from 16 to 37 %, and neurological sequelae, including hearing loss, focal neurological deficits, and cognitive impairment, occurring in 30–52 % of surviving patients.<sup>1–6</sup> Host genetic variation has been shown to influence susceptibility and outcome of pneumococcal and meningococcal infections.<sup>7,8</sup>

The inflammasomes are intracellular signaling complexes belonging to the Nod-like receptors (NLRs).<sup>9–11</sup> To date, four major inflammasome complexes have been described, of which the Nod-like receptor protein 3 (NLRP3) inflammasome has been investigated most extensively.<sup>9–11</sup> The inflammasomes can be activated by several endogenous as well as exogenous danger signals, including ATP, changes in K<sup>+</sup> concentration, oxygen radicals, and uric acid released through cell injury in inflammation.<sup>12,13</sup> Bacterial components with inflammasome-activating properties include bacterial DNA and bacterial toxins. The primary result of inflammasome activation is the binding and activation of caspase-1.<sup>12,13</sup> While several inflammasomes are capable of directly converting the inactive pro-caspase-1 into the active form (e.g., NLRP1 and NLRC4), some (e.g., NLRP3) require the binding of an adaptor protein ASC (adaptor apoptosis-associated speck-like protein). The caspase recruitment domain (CARD) on either NLRP itself or ASC then binds to a CARD domain on the inactive caspase-1, which subsequently can be activated. Active caspase-1 contributes to the conversion of the inactive pro-interleukin-1 beta (pro-IL-1 $\beta$ ) and pro-IL-18 into the respective active and secreted cytokines.<sup>9–11</sup>

One of the key regulators of caspase activity has been shown to be caspase-associated recruitment domain-8 (CARD8), which has been demonstrated to bind the CARD domain of caspase-1 and negatively regulate IL-1 $\beta$  and IL-18 production.<sup>14</sup>

Several findings in patients and animal models suggest a pivotal role for inflammasomes in the pathophysiology of bacterial meningitis.<sup>15</sup> Firstly, in adults with bacterial meningitis, cerebrospinal fluid (CSF) levels of caspase-1 were elevated compared to noninfected patients.<sup>16</sup> Furthermore, in children with pneumococcal meningitis, IL-1 $\beta$  concentrations in the CSF were elevated, a finding that also has been observed in various animal models.<sup>17,18</sup> While the role of IL-1 $\beta$  in the pathogenesis of pneumococcal meningitis has not been elucidated yet, various clinical effects have been attributed to caspase-1, IL-1 $\beta$ , and IL-18-mediated processes.<sup>15,16,18,19</sup> Finally, recent pneumococcal meningitis animal studies showed that lack of the inflammasome components ASC or NLRP3 decreased scores of clinical and histological disease severity in murine pneumococcal meningitis.<sup>19</sup>

We performed a prospective nationwide genetic association study in patients with community-acquired bacterial meningitis to investigate the role of common variants in genes encoding inflammasome components NLRP1, NLRP3, NLRC4, AIM2, PYCARD

(ASC), as well as regulator protein CARD8 on clinical outcome. Subsequently, we determined the principle products of inflammasome activation, IL-1 $\beta$  and IL-18, in the CSF of patients with bacterial meningitis and looked for associations with clinical outcome and the genetic polymorphisms.

## Methods

In a nationwide prospective cohort study, we included bacterial meningitis patients older than 16 years of age with positive CSF cultures who were identified by The Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) from March 2006 to June 2009. The NRLBM provided the names of the hospitals where patients with bacterial meningitis had been admitted 2–6 days previously. The treating physician was contacted, and informed consent was obtained from all participating patients or their legally authorized representatives. Controls for exposure/susceptibility were patients' partners or their nonrelated proxies living in the same dwelling. Data on age, sex, and ethnicity of controls were collected. Secured online case-record forms were used to collect data on patient history, symptoms and signs on admission, treatment, complications, and outcome. Outcome was graded at discharge according to the Glasgow Outcome Scale, a well-validated instrument with good interobserver agreement.<sup>20</sup> A score of 1 on this scale indicates death, a score of 2 a vegetative state, a score of 3 severe disability, a score of 4 moderate disability, and a score of 5 mild or no disability. A favorable outcome was defined as a score of 5, and poor outcome as a score of 1–4. The research ethics committee of the Academic Medical Center approved the study.

### Genotyping

We selected nonsynonymous single-nucleotide polymorphisms (SNPs) in coding regions of genes involved in the inflammasome activation (*NLRP1*, *NLRP3*, *NLRP4*, *PYCARD*, *AIM2*, and *CARD8*) for which a commercial genotyping assay was available and the reported minor allele frequency (MAF) was >5 %. Selected SNPs in *NLRP1* (rs12150220, rs11651270, and rs2301582), *NLRP3* (rs10754558 and rs35829419), *PYCARD* (rs11648861), *AIM2* (rs2276405), and *CARD8* (rs2043211) were genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) in a LightCycler480 (Roche, Basel, Switzerland) using the TaqMan Genotyping Master Mix (Applied Biosystems), at the Department of Genome Analysis in the Academic Medical Center, Amsterdam, The Netherlands. We additionally genotyped one uncommon SNP in *NLRP3* (rs35829479; reported MAF 2.5 %) as it was previously described to interact with the rs2043211 in *CARD8* in inflammatory disease.<sup>21</sup> No assays for common nonsynonymous SNPs in the coding regions of *NLRP4* were commercially available. Laboratory personnel were blinded to clinical information.

### IL-1 $\beta$ and IL-18 measurements in CSF samples of patients with bacterial meningitis

We measured IL-1 $\beta$  and IL-18 in the CSF of patients with bacterial meningitis included in the cohort and 19 control patients with Luminex® technology using a Milliplex assay (Millipore, Billerica, MA, USA). CSF from the first diagnostic tap was collected, centrifuged, and supernatant was aliquoted and stored at  $-80^{\circ}\text{C}$  until analysis. Control CSF was obtained from patients evaluated for acute headache, without signs of meningitis and normal CSF findings. In these patients, a subarachnoid hemorrhage was excluded as cause of their headache by CSF examination. Leftover CSF was collected and centrifuged, and the supernatant was stored at  $-80^{\circ}\text{C}$  until analysis.

### Statistics

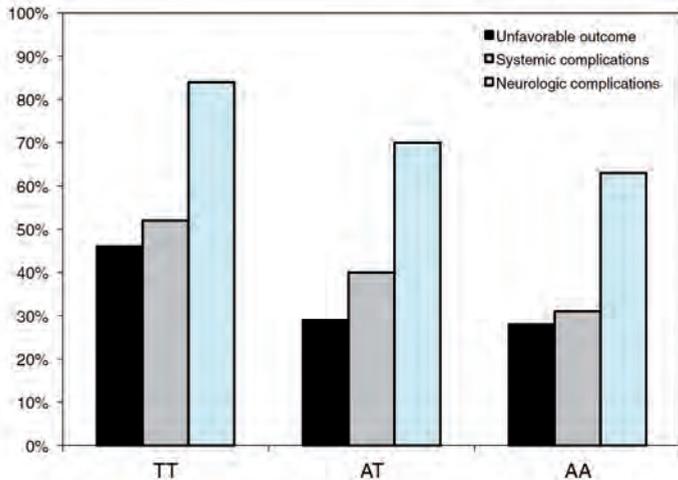
The Mann–Whitney  $U$  test was used to identify differences in baseline characteristics among groups with respect to continuous variables, and dichotomous variables were compared with use of the  $\chi^2$  test. These statistical tests were two-tailed, and  $P < 0.05$  was regarded as significant. Differences in genotype frequencies were analyzed with the  $\chi^2$  or Fishers' exact tests by use of the programs SPSS 19. The main analysis was limited to common SNPs (i.e., minor allele frequencies  $>5\%$ ). For the functional SNP rs2043211 in *CARD8*,  $P < 0.05$  was used to indicate significance. For the other four common SNPs, we performed the analysis both with ( $P < 0.0125$ ) and without ( $P < 0.05$ ) correction for multiple testing. A further analysis was performed to determine the effect of having either a variant allele for rs35829479 (*NLRP3*) or rs2043211 (*CARD8*) on outcome, as this combination was previously described to be associated with inflammatory disease, using  $P < 0.05$  to indicate significance.

We calculated whether the genotype frequencies in the control groups concurred with the Hardy–Weinberg equilibrium (HWE) by use of a  $\chi^2$  and exact test with one degree of freedom. SNPs deviating from the HWE were excluded. The genotype frequencies of patients with a favorable outcome were compared with those with poor outcome as defined by the Glasgow Outcome Scale. Survival data were plotted for the different genotypes using a Kaplan–Meier curve and analyzed using a log rank test. We corrected for possible confounders (age, sex, immunocompromise, and prehospital antibiotic treatment) by performing a multivariate logistic regression analysis including identified polymorphisms and potential confounders. Furthermore, we performed a test of formal interaction of gender and *CARD8* rs2043211 genotype to assess if a gender specific association of this SNP influenced outcome.<sup>22</sup>

## Results

A total of 801 Dutch patients with bacterial meningitis were included as described previously.<sup>23</sup> In this study, the distribution of causative organisms was *S. pneumoniae* in 576 episodes (72%), *Neisseria meningitidis* in 92 (12%), *Listeria monocytogenes* in 41 (5%), and

other bacteria in 92 (12 %) episodes. The case fatality rate was 18 %, and 38 % of patients had poor clinical functional outcome as defined as scores of 1–4 on the Glasgow Outcome Scale. DNA was available for 531 (66 %) patients and 376 controls. Clinical characteristics of this patient population are provided in Table 1.



**Figure 1:** Rate of mortality, systemic, and neurological complications by *CARD8* rs2043211 genotype in pneumococcal meningitis patients. *CARD8* rs2043211 was associated with poor outcome an additive model ( $P=0.040$ ). Patients with the T/T genotype had the highest risk for poor outcome [odds ratio (OR), 2.09; 95 % confidence interval (CI), 1.17–3.71]. This effect on outcome seemed to be driven both by occurrence of systemic (OR T/T genotype, 2.48; 95 % CI, 1.29–4.7;  $p=0.016$ ) and neurological complications ( $p=0.022$ ; OR T/T genotype, 3.03; 95 % CI, 1.34–6.85).

Genotyping success rate was >95 % for all assays. Three SNPs that were uncommon (*PYCARD*rs11648861) or monomorphic (*NLRP3* rs35829419, and *AIM2* rs2276405) were excluded from the analysis. The genotype frequency concurred with the Hardy–Weinberg equilibrium in the control population for all SNPs. We identified rs2043211 in *CARD8* to be associated with poor outcome of bacterial meningitis using an additive model ( $p=0.040$ ; Table 2). Patients with the T/T genotype had the highest risk for poor outcome [odds ratio (OR), 2.09; 95 % confidence interval (CI), 1.17–3.71;  $p=0.009$ ]. In a multivariate analysis limited to white patients, rs2403211 was an independent risk factor for unfavorable outcome after correction for age, sex, causative bacteria, immunodeficiency, and pretreatment with antibiotics (OR, 2.10; 95 % CI, 1.04–4.21;  $p=0.038$ ). The effect of rs2043211 was stronger in the subgroup of white patients with pneumococcal meningitis (Fig. 1; OR for T/T genotype, 2.19; 95 % CI, 1.15–4.18;  $p=0.018$ ). This effect on outcome seemed to be driven both by occurrence of systemic (OR T/T genotype, 2.48; 95 % CI, 1.29–4.7;  $p=0.016$ ) and neurological complications (OR T/T genotype, 3.03; 95 % CI, 1.34–6.85;  $p=0.022$ ). When testing the equality of the genotype versus outcome odds ratios in men and women, we could not demonstrate a statistically significant interaction between genotype and gender. Patients with either a variant allele for *CARD8* rs2043211 or *NLRP3* rs35829419, which

was previously described to cause a deficient phenotype, were not at increased risk for death, unfavorable outcome, or complications. Rs11651270 (Met1154Val) in *NLRP1* was associated with death in pneumococcal meningitis patients using a recessive model (14 % TT genotype vs. 6 % CC/CT genotype; OR, 1.97; 95 % CI, 1.01–3.85,  $p=0.047$ ; log rank survival analysis  $p=0.04$ , Fig. 2). After correction for age, sex, immunodeficiency, and pretreatment with antibiotics, the effect of rs11651270 on mortality remained significant (OR, 2.32; 95 % CI, 1.12–4.78;  $p=0.023$ ). Using a Bonferroni correction, the effect of rs11651270 on death was no longer significant. Other SNPs in *NLRP1* and *NLRP3* were not associated with outcome or death. CSF was obtained from 289 patients with bacterial meningitis and 19 control patients. Levels of IL-1 $\beta$  and IL-18 were elevated in the CSF of patients with bacterial meningitis as compared to controls [median, 1.31 ng/ml (IQR, 0.19–4.40) vs. 0.004 ng/ml (IQR, 0.002–0.006),  $p<0.001$ , and 10.76 ng/ml (IQR, 4.00–25.02) vs. 0.71 ng/ml (IQR, 0.40–0.89),  $p<0.001$  respectively]. High IL-1 $\beta$  levels were associated with occurrence of systemic complications [Fig. 3; median, 1.94 ng/ml (IQR, 0.30–5.26) vs. 0.93 ng/ml (IQR, 0.15–3.11),  $p=0.003$ ]. There was a trend between high IL-1 $\beta$  levels and neurological complications [median, 1.62 ng/ml (IQR, 0.28–5.04) vs. 0.43 ng/ml (IQR, 0.08–4.73),  $p=0.10$ ], as well as unfavorable outcome [median, 1.53 ng/ml (IQR, 0.28–5.19) vs. 1.03 ng/ml (IQR, 0.17–3.63),  $p=0.08$ ]. High IL-18 levels were also associated with systemic complications [Fig. 3; median, 15.13 ng/ml (IQR, 6.36–26.89) vs. 8.84 ng/ml (IQR, 3.09–19.91),  $p=0.004$ ] and poor outcome [median, 14.48 ng/ml (IQR, 5.26–26.59)

**Table 1:** Clinical characteristics of 531 patients with community acquired bacterial meningitis [data are number/number evaluated (percentage) or mean  $\pm$  SD].

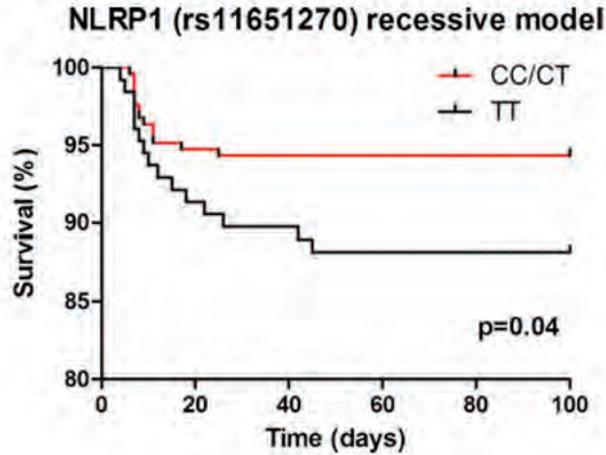
Characteristic	Value/Total	Characteristic	Value/Total
Age (year)	55 $\pm$ 17	Indexes of CSF inflammation <sup>b</sup>	
Male sex	262 (49%)	Opening pressure (mmH <sub>2</sub> O)	34 $\pm$ 11
Pre-treatment with antibiotics	63/527 (12%)	WBC (/mm <sup>3</sup> )	6778 $\pm$ 13319
Predisposing conditions	227 (43%)	WBC < 1,000/mm <sup>3</sup>	142/496 (27%)
Otitis or sinusitis	191 (36%)	Protein (g/L)	4.3 $\pm$ 3.0
Pneumonia	77 (15%)	CSF blood: glucose ratio	0.14 $\pm$ 0.19
Immunocompromise	124 (23%)	Positive blood cultures	346/463 (75%)
Symptoms at presentation		Complications	
Headache	411/479 (85%)	Systemic complications	166 (31%)
Neck stiffness	398/510 (78%)	Neurologic complications	327 (62%)
Systolic blood pressure (mmHg)	146 $\pm$ 29	Glasgow outcome scale	
Heart rate (bpm)	99 $\pm$ 21	1 – Death	40/528 (8%)
Body temperature (°C)	38.7 $\pm$ 1.3	2 – Vegetative state	1/528 (0.2%)
Score on Glasgow coma scale <sup>a</sup>	11 $\pm$ 3	3 – Severe disability	21/528 (4%)
< 8 indicating coma	70/527 (13%)	4 – Moderate disability	78/528 (15%)
Focal neurologic deficits	141/528 (27%)	5 – Good recovery	388/528 (73%)

<sup>a</sup>Glasgow coma scale score was evaluated in 527 patients. <sup>b</sup>CSF pressure was evaluated in 123 patients, CSF WBC in 496, CSF protein in 505, and CSF blood to glucose ratio in 498.

**Table 2:** Genotyping results of six genetic polymorphisms in inflammasome genes in 531 bacterial meningitis patients of which 388 patients with a good outcome and 143 with poor outcome. <sup>a</sup>Glasgow Outcome Scale Score.

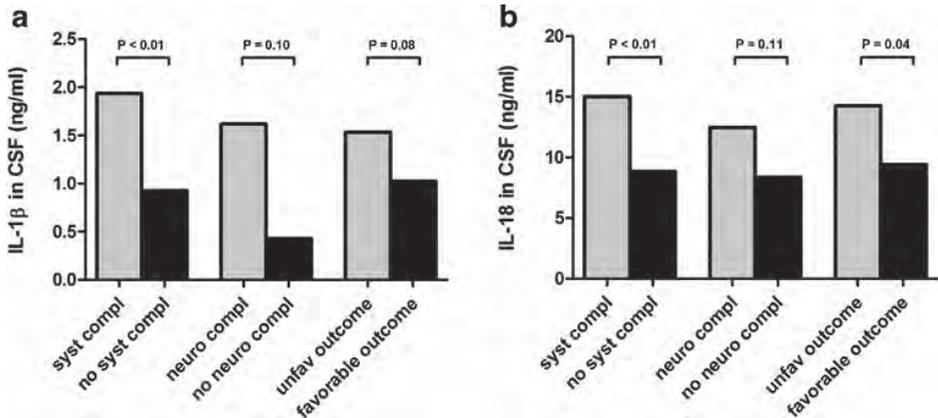
Gene	SNP ID	Good outcome (GOS 5)*			Poor outcome (GOS 1-4)			Model	P-value				
		A	B	AA	AB	BB	A			B	AA	AB	BB
CARD8	rs2043211	525	237	183	159	39	169	107	57	55	26	Additive	0.04
NLRP1	rs12150220	433	327	127	179	74	173	103	57	59	22	Recessive	0.21
NLRP1	rs11651270	406	356	105	196	80	165	109	51	63	23	Recessive	0.12
NLRP1	rs2301582	473	299	143	187	56	183	97	58	67	15	Recessive	0.14
NLRP3	rs10754558	433	331	120	193	69	161	119	49	63	28	Recessive	0.65
NLRP3	Rs35829419	701	35	334	33	1	227	7	110	7	0	Dominant	0.43

\*Glasgow outcome scale score



**Figure 2:** Kaplan–Meier survival curve in patients with pneumococcal meningitis according to rs11651270 genotype.

vs. 9.43 ng/ml (IQR, 3.37–22.68),  $p = 0.039$ ]. In the subgroup of patients with pneumococcal meningitis ( $n = 207$ ), associations with systemic complications remained significant. CSF levels of IL-1 $\beta$  and IL-18 between patients were not associated with rs2043211 and rs11621270 genotypes, also after a correction for total CSF protein levels was applied.



**Figure 3:** Median levels of IL-1 $\beta$  and IL-18 in CSF of patients with bacterial meningitis. a Elevated IL-1 $\beta$  was associated with systemic complications, and there was trend towards more neurological complications. b High IL-18 levels were associated with both systemic complications and unfavorable outcome.

## Discussion

Our results implicate an important role of the inflammasomes in bacterial meningitis. We found that SNPs in the inflammasome genes *CARD8* (rs2043211) and *NLRP1* (rs11621270) are associated with death and poor disease outcome. IL-1 $\beta$  and IL-18 levels in CSF of

patients with bacterial meningitis correlated with development of systemic complications and poor prognosis.

We identified the rs2043211 polymorphism in *CARD8* to contribute to outcome of bacterial meningitis by influencing the risk of systemic complications. The A allele of rs2043211 generates a premature stop codon (Cys10X) and leads to a severely truncated *CARD8* protein, which has been associated with various inflammatory diseases such as inflammatory bowel disease, Crohn's disease, and rheumatoid arthritis.<sup>21,22,24-26</sup> Several functions have been attributed to *CARD8*. First is the inhibition of pathways to nuclear factor kappa B (NF- $\kappa$ B) activation.<sup>27,28</sup> In vitro studies have demonstrated that *CARD8* interferes with NF- $\kappa$ B activation by established NF- $\kappa$ B activators, possibly through direct interaction between *CARD8* and I $\kappa$ B kinase complex.<sup>27</sup> Second, *CARD8* also has antiapoptotic properties through the inhibition of caspases, including caspase-1, caspase-8, and caspase-9 (*CARD8* is also known as TUNCAN, tumor-upregulated *CARD* containing antagonist of caspase-9).<sup>29</sup> Through direct interaction with caspase-1, *CARD8* can negatively regulate caspase-1 dependent IL-1 $\beta$  generation in vitro.<sup>14</sup> Lastly, *CARD8* forms a physical component of the multiprotein complex of the NLRP3 inflammasome.<sup>30,31</sup> However, in vitro knockdown studies have shown that *CARD8* may not be a requirement for the activation of the NLRP3 inflammasome in response to viral infection.<sup>32</sup>

The truncated form of *CARD8*, therefore, has the potential to disrupt cytokine regulation at several key stages and could lead to higher levels of NF- $\kappa$ B mediated proinflammatory (pro)cytokines, incomplete NLRP3 assembly, and a limited caspase-1 activation, resulting in limited secretion of activated IL-1 $\beta$  and IL-18. Conversely, the normal form of *CARD8* (T/T genotype) may lead to NF- $\kappa$ B inhibition and lower proinflammatory (pro)cytokines. Despite proper NLRP3 assembly with *CARD8*, this could *also* lead to limited secretion of IL-1 $\beta$  and IL-18. Indeed, we do not see a difference in CSF levels of IL-1 $\beta$  and IL-18 between *CARD8* genotypes. The suppressed noncaspase-dependent inflammation may, however, be insufficient to battle bacterial infection, resulting in the observed increased risk of systemic and neurological complications in patients with bacterial meningitis.

Our findings support the hypothesis that genetic variation in the inflammasome genes can influence the threshold for activation of the inflammatory response, presenting a double-edged sword: A more readily activated system will predispose to chronic inflammation (rheumatoid arthritis and inflammatory bowel disease), while a normally controlled system may result in suboptimal activation and less control of severe infection (bacterial meningitis).

We identified rs11651270 SNP in *NLRP1* to influence mortality in bacterial meningitis, although the effect was no longer significant after correction for multiple testing. The exact function of *NLRP1* remains unclear, though its relevance is underlined by associations between SNPs in *NLRP1* and autoimmune diseases such as vitiligo, autoimmune Addison's disease, type 1 diabetes, and Alzheimer's disease.<sup>12,33,34</sup> To our knowledge, this is the first report of rs11651270 to be associated the outcome of infectious disease. As the effect of

rs11651270 was not significant after correction for multiple testing, this result should be regarded as explorative and needs validation in other populations before a firm conclusion can be drawn.

NLRP1 is activated by two known factors: anthrax lethal toxin derived from spore-forming bacterium *Bacillus anthracis*, and muramyl dipeptide, a peptidoglycan constituent of both Gram-positive and Gram-negative bacteria.<sup>35</sup> Unlike NLRP3, NLRP1 has its own CARD domain and does not require ASC or CARD8 to activate caspase-1 (although the presence of ASC substantially increases caspase activation).<sup>13</sup> However, as the rs11651270 polymorphism does not seem to influence levels of IL-1 $\beta$  or IL-18 in the CSF of our patients, a caspase-dependent mechanism does not seem likely.

NLRP1 and CARD8 share a “function-to-find domain” (FIIND), which is a highly conserved domain only present in these two proteins. FIIND has an intraproteolytic function, of which the relevance is incompletely understood.<sup>36</sup> Interestingly, the aforementioned NLRP1 SNP lies *in*, and the CARD8 SNP is situated *before* the respective FIIND regions.<sup>36</sup> Although the influence of rs11651270 on the function of FIIND is unknown, one can hypothesize that a disruption of the FIIND domain could affect NLRP1 function and thereby influence clinical outcome following bacterial meningitis.

IL-1 $\beta$  and IL-18 levels in CSF were found to correlate with outcome, but were not associated with the polymorphisms in *NLRP1* or *CARD8*. A possible explanation for this discrepancy could be that IL-1 $\beta$  and IL-18 are also being produced in an inflammasome-independent manner. This was previously shown for IL-1 $\beta$ , which can be produced by neutrophil-derived serine proteases or pathogen-derived proteases.<sup>37</sup> Therefore, a small potential decrease in cytokine production due to *NLRP1* and *CARD8* polymorphisms may not be measurable in the total amount of IL-1 $\beta$  and IL-18 produced. Another explanation may be that the impact of these polymorphisms on secreted active IL-1 $\beta$  and IL-18 levels is limited, and *NLRP1* and *CARD8* may be involved in alternative inflammatory roles.<sup>37</sup> Further functional studies of rs2043211 and rs11621270 are needed determine the influence on these SNPs on the immune response after stimulation with *S. pneumoniae*.

Our study has several limitations. First, our findings regarding the *NLRP1* and *CARD8* SNPs should be replicated in independent case-control study to validate our observations. However, currently, no such studies are available for us to confirm our findings. Second, in this study, we show an association between the polymorphisms rs2043211 and rs11621270, and poor outcome and death, but we did not demonstrate changes in protein functionality or a causal relationship with outcome. Once the associations have been confirmed, further mechanistic studies of the functionality of these SNPs during infection should be performed.

In conclusion, our results implicate an important role of genetic variation in inflammasome genes in bacterial meningitis. Interference with inflammasome activation may therefore be a promising target for adjunctive therapy in bacterial meningitis.

## Declarations

### Author Contributions

The work presented here was carried out in collaboration between all authors. All authors have read and approved the final version of the manuscript.

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### Potential conflicts of interest

The authors have no conflicts of interest to report.

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# 6

## Streptococcus pneumoniae arginine synthesis genes promote growth and virulence in pneumococcal meningitis

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## Abstract

*Streptococcus pneumoniae* (pneumococcus) is a major human pathogen causing pneumonia, sepsis and bacterial meningitis. Using a clinical phenotype based approach with bacterial whole-genome sequencing we identified pneumococcal arginine biosynthesis genes to be associated with outcome in patients with pneumococcal meningitis. Pneumococci harboring these genes show increased growth in human blood and cerebrospinal fluid (CSF). Mouse models of meningitis and pneumonia showed that pneumococcal strains without arginine biosynthesis genes were attenuated in growth or cleared, from lung, blood and CSF. Thus, *S. pneumoniae* arginine synthesis genes promote growth and virulence in invasive pneumococcal disease.

## Introduction

*Streptococcus pneumoniae* can cause a range of invasive infections, such as pneumonia and meningitis, with devastating consequences worldwide.<sup>1-3</sup> Pneumococcal meningitis continues to exact a heavy toll despite the implementation of childhood vaccination programs.<sup>4,5</sup> The fatality rate in patients with pneumococcal meningitis is substantial, at 30%, and long-term sequelae, including hearing loss, focal neurological deficit, and cognitive impairment, develop in about half of survivors.<sup>3,6</sup>

Invasive disease caused by *S. pneumoniae* is seen at the beginning and end of life or in patients with underlying conditions.<sup>1,4</sup> Several studies of extreme phenotypes have identified genetic defects in the complement system and intracellular signaling proteins to be associated with increased susceptibility<sup>7</sup>. A common variant in complement component 5 has been associated with unfavorable outcome in pneumococcal meningitis<sup>8</sup>. Bacterial genetic factors may also determine susceptibility and disease severity. Few studies have investigated the role of bacterial factors in invasive pneumococcal disease, including meningitis. Pneumococcal capsular polysaccharides have been associated with disease severity and are used for phenotypical characterization<sup>9</sup>. The pneumococcal capsular phenotype has been linked to genotype but the mechanisms of virulence have not been elucidated.<sup>10</sup> *S. pneumoniae* contains an inducible system for the uptake of DNA from the environment that readily allows for horizontal gene transfer.<sup>11</sup> The pneumococcal pan-genome has been estimated at 5000 genes, but less than 50% of these genes are shared by all pneumococcal strains (forming the pneumococcal core genome).<sup>12</sup>

Using a clinical phenotype based approach with bacterial whole-genome sequencing we identified pneumococcal arginine biosynthesis genes to be associated with outcome in patients with pneumococcal meningitis. We found that pneumococci harboring these genes show increased growth in human blood and cerebrospinal fluid. Mouse models of meningitis and pneumonia showed that pneumococcal strains without arginine biosynthesis genes were attenuated in growth and/or cleared from lung, blood and cerebrospinal fluid.

## Methods

### Clinical cohort

The Dutch Meningitis Cohort Studies from 1998–2002 and 2006–2009 are prospective nationwide cohort studies of adults with community-acquired bacterial meningitis in the Netherlands.<sup>3,8</sup> Patients older than 16 years with positive cerebrospinal fluid (CSF) cultures were included after identification by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM). The NRLBM receives isolates from CSF of 90% of all patients with bacterial meningitis in the Netherlands.<sup>3</sup> Written informed consent was obtained from all patients or their legally authorized representatives. Details on the data collection of

the cohorts were presented elsewhere.<sup>3,8</sup> The studies were approved by the Medical Ethics Committee, Academic Medical Center (AMC).

### **Bacterial isolates**

Upon receipt by the NRLBM, isolates were serotyped and stored at  $-80^{\circ}\text{C}$ . Patients' isolates had less than 5 passages before storage. Pneumococci were recultured on Trypticase Soya agar (TSA) (Difco Laboratories, Inc., Detroit, MI), with 5% defibrinated sheep blood.

### **Whole genome sequencing and annotation**

Bacterial genome Roche/454 shotgun sequencing was performed according to the manufacturer's instructions. The mean genomic coverage was 25-fold (range 14–63; Table 1). Based on de novo assemblies, a median of 88 contigs was produced. Sequence reads were sorted by sample and assembled using the Roche GS De Novo Assembler (Newbler) v 2.3 and the Roche GS Reference Mapper v 2.0.0.12. In the de novo assemblies, we allowed 0 and 2 errors in the MID extraction settings. Each of the 20 genomes was also assembled in a reference mapping assembly using 11 publically available pneumococcal reference genomes (Supplementary Table 1). In the Reference Mapper assemblies, 0 errors were allowed in the MID calling parameters. Alignments of the contigs were created with Codoncode Aligner software (version 3.5.1, CodonCode Corporation, Dedham, MA). All contigs were put in a more correct order by rearranging them with the 'move contigs' option in Mauve software using *S. pneumoniae* TIGR4 strain as a reference.<sup>13</sup> In an exploratory analysis of 30 contig boundary regions in 8 pneumococcal genomes, 21 (70%) encoded genes that occurred multiple times in the pneumococcal genome. The median contig-number produced by genome assembly was 27 (range, 13–66). The GC content of sequenced genomes was highly similar, ranging from 39.5% to 39.8%, and genome sizes ranged from 1.98–2.15Mb.

The genomes were annotated using Annotation Engine at the Institute for Genome Sciences of the University of Maryland School of Medicine (<http://ae.igs.umaryland.edu/cgi>). To compare gene content of bacterial isolates from patients with favorable outcome versus isolates from patients who died, we first build an extended genome that represents a non-redundant collection of all genes occurring at least once in our 20 genomes. We started collection with genes of genome AMCSP01 and aligned all genes of all genomes (one by one) to this collection using Blast Like Alignment Tool (BLAT).<sup>14</sup> Genes were added to the extended genome when they did not match to the extended genome that was build so far. The matching criteria were set to 90% identity and 90% query coverage.

### **Genome comparison and validation**

Genes of the extended genome were aligned to genomes of all bacteria. We scored per bacteria which genes were present with similar BLAT matching criteria. As positive control, we used an artificial gene that was correctly identified in all genomes (row 2 Supplementary Table 2). Candidate gene clusters were evaluated in duplicate by PCR

with primers specific for one gene in the cluster (Supplementary Table 3). The *argGH* locus was determined by PCR using 2 primer sets specific for either the *argG* or *argH* gene (Supplementary Table 3).

#### **Pneumococcal knock-out strain**

The D39 *argGH* knock out mutant was constructed as described by Trzciński.<sup>15</sup> A Janus-type cassette with kanamycin resistance and streptomycin sensitivity alleles with flanking DNA sequences corresponding to sequences that flank the *argGH* locus, was constructed using a 1368-bp fragment containing *aphIII-rpsL* amplified from chromosomal DNA of TIGR4 $\Delta$ pspA (Supplementary Table 3).<sup>16,17</sup> Flanking sequences, one with a BamHI 3' terminus and one with an ApaI 5' terminus, were amplified using chromosomal DNA of D39 as template and primer pairs KSPNArg2061617F8/ KSPNArg2061617R6 and KSPNArg2061617F7/KSPNArg2061617R9\_1. The *aphIII-rpsL* and flanking sequence PCR products were digested with BamHI and ApaI, and purified with a GeneJET Gel Extraction Kit (Fermentas). The digested PCR products were ligated using T4 DNA ligase (Roche) and the ligation mixture was used as template in a PCR with primers KSPNArg2061617F1 and KSPN2061617ArgR9\_1. The 2525 bp PCR product was used to transform D39 with selection for resistance to kanamycin (Km<sup>R</sup>) to create D39  $\Delta$ *argGH*. The structure of the insertion was confirmed by PCR and sequencing.

#### **Bacterial growth in blood**

Five pneumococcal colonies from an overnight culture on TSA were collected and resuspended in 5 mL Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) and grown to optical density at 450 nm (OD<sub>450</sub>) between 0.25 and 0.35. Bacteria were washed with nutrient mixture F-10 Ham (Sigma-Aldrich) and resuspended in 5 mL F-10 Ham supplemented with citrulline or arginine where appropriate to OD at 620 nm of 0.01. Hundred  $\mu$ L bacterial suspension was mixed with 100  $\mu$ L citrate blood or 100  $\mu$ L CSF and incubated for 6 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

#### **Exposure to oxidative stress**

Bacteria were grown to an OD<sub>450</sub> of 0.15 in THY. Cultures were washed with THY and 100-fold diluted in THY or in THY with 0.04% H<sub>2</sub>O<sub>2</sub>. Cultures were incubated without agitation at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. At t = 0, 30, 60, 90 and 120 minutes, samples were taken and serially diluted for CFU count.

**Table 1:** 20 pneumococcal strains from patients with pneumococcal meningitis.

Strain	Clinical characteristics					Pneumococcal typing		
	Sex	Age	GCS	CSF WBC	GOS	Serotype	Sequence type	Clonal complex
AMCSP01	M	76	13	46	1	22F	433	433
AMCSP02	M	68	10	1,877	1	20	1030	235
AMCSP03	M	64	11	5,200	1	10A	97	460
AMCSP04	F	67	9	267	1	7F	191	191
AMCSP05	M	60	10	40	1	9V	2726	156
AMCSP06	M	57	15	7,230	1	23F	507	439
AMCSP07	M	58	13	12,400	1	23F	440	156
AMCSP08	F	30	14	2,000	1	18C	1233	1381
AMCSP09	M	61	11	592	1	4	247	246
AMCSP10	M	82	9	2,810	1	8	53	53
AMCSP11	F	60	8	2,220	5	23F	507	439
AMCSP12	M	62	8	3,307	5	7F	191	191
AMCSP13	M	37	15	2,747	5	18C	113	113
AMCSP14	M	60	15	3,960	5	9V	162	156
AMCSP15	M	88	15	1,700	5	8	53	53
AMCSP16	F	69	13	5,000	5	10A	1551	460
AMCSP17	M	54	9	35,000	5	23F	36	439
AMCSP18	F	52	7	12	5	4	205	205
AMCSP19	M	50	15	3,000	5	20	235	235
AMCSP20	M	67	12	48	5	22F	433	433

CSF WBC (cells/mm<sup>3</sup>) denotes cerebrospinal fluid white blood cells, GCS Glasgow Coma Scale, GOS Glasgow Outcome Scale. Sequence Type was based on Multi Locus Sequence Typing (MLST).<sup>10</sup>

## RT PCR

Bacteria were grown to an OD<sub>450</sub> of 0.2 in THY. Bacteria were collected by centrifugation and resuspended in PBS and thereafter an enzyme cocktail of Achromopeptidase (1000 U/mL), Mutanolysine (100 U/mL) and Lysozym (10 mg/mL) was added and incubated for 30 minutes at 37°C. Bacteria were collected by centrifugation and the pellet was resuspended in Tris-EDTA buffer with 1 mg/mL lysozyme and RNA was isolated using the RNeasy midikit (QIAgen), according to the manufacturer's protocol. RNA was treated with the Turbo DNase (AMBION) according to the manufacturer's protocol and cDNA was made with the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis kit (Life technologies). Primers are shown in Supplementary Table 3.

## Mouse models of meningitis and pneumonia

Mouse experiments were conducted with specific pathogen-free male C57BL/6 mice (Charles River, Wilmington, MN, USA) aged 8–12 weeks. Experiments were approved by the Institutional Animal Care and Use Committee, AMC.

For the induction of meningitis, D39 and D39  $\Delta argGH$  were grown to mid log phase at 37°C in Todd-Hewitt broth supplemented with yeast (THY, Difco, Detroit, MI) and

harvested by centrifugation. Pneumococci were diluted with sterile saline to an OD<sub>620</sub> of 0.3 corresponding to  $1 \times 10^8$  CFU mL<sup>-1</sup>. Inoculum dose was based on a previous study.<sup>18</sup> Twelve mice per group were inoculated into the cisterna magna under isoflurane anesthesia, with 1  $\mu$ L bacterial suspension containing D39 or D39 $\Delta$ *argGH*, as described earlier.<sup>19</sup>

For the induction of pneumonia D39 and D39  $\Delta$ *argGH* were grown to mid log phase at 37°C in Todd-Hewitt broth supplemented with yeast (Difco, Detroit, MI) and harvested by centrifugation. Eight mice per group were intranasally inoculated with D39 or D39  $\Delta$ *argGH* by putting a 50  $\mu$ L of  $5 \times 10^8$  CFU per mL droplet of bacterial suspension on the nose under isoflurane anesthesia, as described previously.<sup>20</sup>

### Statistics

For analysis of survival data the Log-rank test was used. The Mann–Whitney U test was used to identify differences between patient and animal groups with respect to continuous variables, and dichotomous variables were compared with use of the  $\chi^2$  test. We used logistic regression analysis to calculate odds ratios (OR) and 95% confidence intervals (CI). All statistical tests were two-tailed;  $P < 0.05$  was regarded significant.

## Results

### Pneumococcal gene cluster associated with outcome in patients with meningitis

From 2006 through 2009, we included 642 episodes of community-acquired bacterial meningitis in a prospective nationwide cohort<sup>8</sup>. Overall, case fatality rate was 19% and 39% patients had an unfavorable outcome, defined as a score of 1–4 on the Glasgow outcome scale. 427 of these patients had pneumococcal meningitis. In an extreme phenotype approach, we sequenced genomes of 10 randomly selected pneumococcal isolates from immunocompetent patients who died and 10 isolates from matched patients with favorable outcome (patients were matched for serotype, age, absence of predisposing factors; Table 2). Genomes were annotated resulting in a median of 2190 open reading frames per genome (range, 2054 to 2882; Table 1 and Supplementary Table 1). The number of genes present in all sequenced genomes, the pneumococcal core genome, consisted of 1981 of 3453 genes (57%; 1.55 Mb, Supplementary Table 2), is consistent with previous work.<sup>13</sup> Genes ( $n = 2652$ ) that were equally represented in both groups, and genes found only once per group were not further analyzed (Figure 1A; 642 genes were included; 273 pneumococcal genes associated with death, and 369 associated with favorable outcome). Next, four genes were excluded from the analysis since only a partial sequence was available. Finally, 86 of 155 (55%) genes with a group frequency difference  $\geq 2$  were located in 14 gene clusters: 5 clusters including 28 genes were associated with favorable outcome, and 9 clusters including 58 genes were associated with unfavorable outcome (Figure 1B).

Subsequently, we evaluated associations between 14 gene clusters and clinical outcome in 748 other new pneumococcal meningitis patients included in two prospective

nationwide cohort studies.<sup>3,8</sup> In the first cohort (321 pneumococcal strains, Table 3) three gene clusters were significantly associated with favorable outcome. In the second cohort (427 pneumococcal strains), used as a subsequent validation cohort, Cluster L remained significantly associated with favorable outcome ( $P = .027$ ).

**Table 2:** Whole Genome Sequencing Results for 20 Pneumococcal Strains From Patients With Community-acquired Bacterial Meningitis.

Bacterial genome	Sequenced nucleotides (Mb)	Sequence depth <sup>a</sup>	Coding sequence (%)	Contigs	Genome size (Mb)	GC content (%)	No. genes
AMCSP01	35	17	86.4	28	2.07	39.5	2,196
AMCSP02	72	35	86.2	23	2.08	39.6	2,175
AMCSP03	45	22	86.3	24	2.05	39.6	2,133
AMCSP04	62	30	86.1	31	2.00	39.7	2,074
AMCSP05	33	16	86.0	66	2.15	39.5	2,280
AMCSP06	60	29	85.4	33	2.05	39.6	2,308
AMCSP07	68	33	86.4	25	2.11	39.6	2,215
AMCSP08	32	16	85.2	37	2.07	39.7	2,437
AMCSP09	28	14	86.1	26	2.06	39.6	2,234
AMCSP10	48	24	86.2	13	1.98	39.7	2,054
AMCSP11	39	19	86.0	37	2.04	39.6	2,151
AMCSP12	65	32	85.1	31	2.00	39.8	2,160
AMCSP13	84	41	82.7	41	2.16	39.6	2,882
AMCSP14	57	28	86.1	53	2.14	39.5	2,275
AMCSP15	30	15	86.0	22	2.03	39.7	2,161
AMCSP16	54	26	86.3	27	2.06	39.6	2,168
AMCSP17	130	63	86.2	22	2.07	39.7	2,183
AMCSP18	48	24	86.2	26	2.14	39.5	2,312
AMCSP19	57	28	85.9	27	2.05	39.5	2,160
AMCSP20	37	18	86.4	44	2.14	39.5	2,382

<sup>a</sup>Sequence depth: the n-fold genome coverage is shown as defined by the sequenced nucleotides divided by the average genome size (2.1 Mb).

### Genomic locus is variable region for genes encoding pneumococcal arginine synthesis

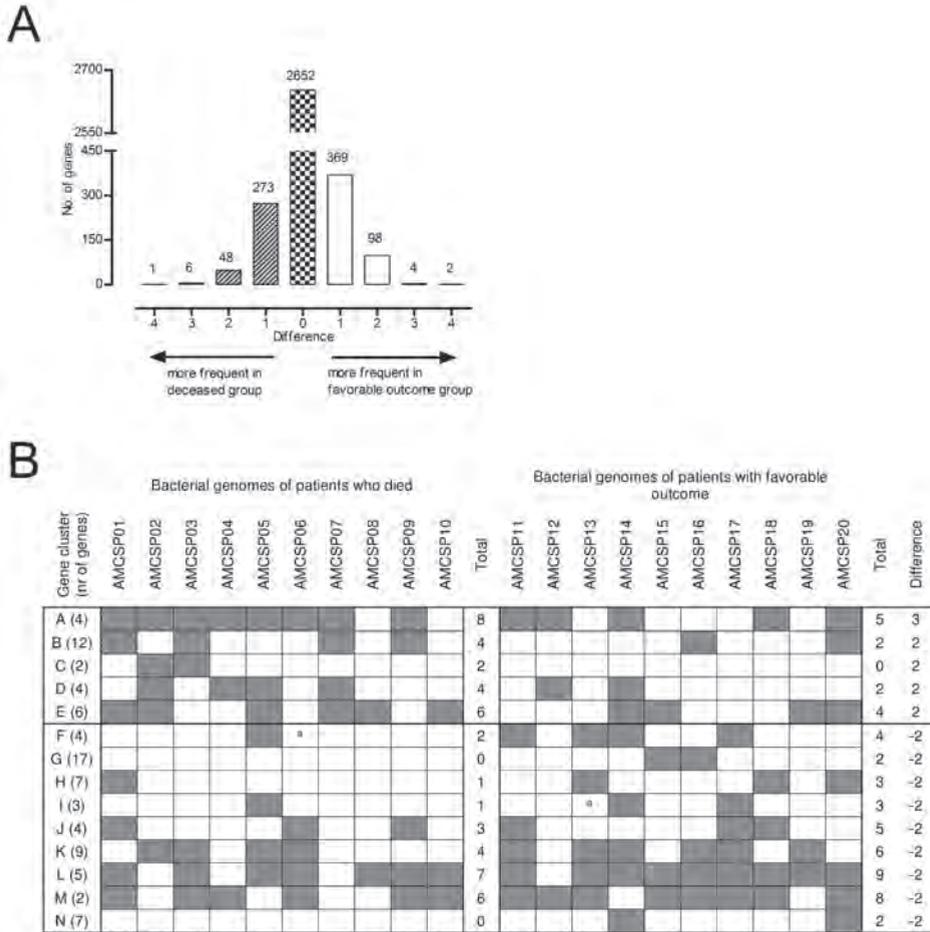
Cluster L (4.4 kb) includes 6 genes that all encode membrane proteins.<sup>21</sup> BLASTX analysis of these genes showed similarities to pneumococcal proteins. The GC content of cluster L was lower than the average of the whole genome (30% versus 40%) and the dinucleotide composition ( $\delta^*$ , genome signature) was highly dissimilar as compared to similar sized fragments of the whole genome (98% of the genome fragments yielded a lower  $\delta^*$  than cluster L).<sup>22</sup> suggesting acquisition by horizontal gene transfer.<sup>23</sup> Cluster L replaced or truncated an *arg* locus comprising genes encoding argininosuccinate synthase (ArgG) and argininosuccinate lyase (ArgH). ArgG converts citrulline together with aspartate to argininosuccinate that is converted to arginine and fumarate by ArgH.<sup>24</sup>

### Genes involved in conversion of citrulline to arginine contribute to pneumococcal growth

Arginine biosynthesis is linked to the biosynthesis of polyamines (Figure 2A), which are implicated to play a role in the resistance to oxidative stress.<sup>25</sup> To gain further insight in the role of the *argGH* locus we used *S. pneumoniae* D39 that contains the *argGH* locus (Genbank accession number NC\_008533.1) and constructed an *argGH* knock out.<sup>26</sup> In standard rich laboratory medium THY D39 and D39 $\Delta$ *argGH* showed equal growth rates (Figure 2B). However, D39 wt was more resistant to 0.04% H<sub>2</sub>O<sub>2</sub> compared to D39 $\Delta$ *argGH* (Figure 2C). In blood CFUs of D39 increased 300- fold after 6 hours incubation, but CFUs of D39 $\Delta$ *argGH* remained at the level of the start inoculum in nonsupplemented blood (Figure 2D). Relative bacterial outgrowth was similar in CSF when compared to blood, although bacterial growth of D39 and D39 $\Delta$ *argGH* did not increase in CSF when 0.25% citrulline was added (Figure 2E). To investigate the role of NO we repeated growth experiments with NO synthetase blockers, but this did not influence growth. *ArgGH* knock outs obtained in two independent transformations had the same phenotype. We did not succeed to complement D39 $\Delta$ *argGH*. In this strain, we assessed transcription of the genes upstream and downstream of the *argGH* locus by RT-PCR, to exclude potential polar effect of its replacement by the Janus cassette. In both D39 wt and D39 $\Delta$ *argGH*, transcription from gene SP\_0112 (encoding a hypothetical protein) downstream of *argGH* was high and comparable to that of *gki* (encoding the housekeeping protein glucose kinase). Transcription from the gene upstream of *argGH* (SPD\_109 encoding a amino acid ABC transporter periplasmic amino acid-binding protein) was detectable in both D39 wt and D39 $\Delta$ *argGH*, albeit lower in the *argGH* knockout strain (results not shown).

### Pneumococcal arginine synthesis contributes to bacterial growth and virulence in mouse models of meningitis and pneumonia

To gain further insight into the role that genes involved in conversion of citrulline to arginine plays in the infectivity and virulence of *S. pneumoniae*, C57BL/6 mice were injected intracisternally with  $1 \times 10^5$ CFU D39 or D39 $\Delta$ *argGH*. Mice with pneumococcal meningitis caused by D39 died within 4 days, whereas all mice infected with D39 $\Delta$ *argGH* survived (log-rank test,  $P < .001$ ; Figure 3A). Subsequently, groups of mice were infected and sacrificed at 6 hours and 40 hours after infection. Organs from both groups of mice were surveyed for viable *S. pneumoniae* CFUs. At 6 hours, CFUs in CSE, blood, and lung were higher in the group infected with D39 compared to the group infected with D39 $\Delta$ *argGH* ( $P < .01$ ; Figure 3B). At 40 hours post-infection, CFUs were higher CSE, brain, blood, and lung in the group infected with wild-type bacteria ( $P < .001$ ; Figure 3B). To investigate the role of arginine synthesis in pneumococcal pneumonia, we inoculated mice intranasally with  $2.5 \times 10^7$  CFU D39 or D39 $\Delta$ *argGH*. At 6 hours post-infection, CFUs were higher in the lung in the group infected with wild-type bacteria ( $P < .001$ ; Figure 3C). At later time points (24 and 48 hours) both D39 and D39 $\Delta$ *argGH* bacteria were cleared from all organs in all infected mice.



**Figure 1:** A, Genomic comparison in a clinical-based approach with whole bacterial genome sequencing of 20 *S. pneumoniae* clinical isolates: differences in gene content between extreme phenotype groups. 328 genes were relatively more present in strains from patients who died (striped) and 473 genes were more present in strains from survivors with favorable outcome (white); 2652 genes were equally represented in both groups. B, Genome comparisons of gene cluster frequency among 20 pneumococcal strains. Gray boxes indicate presence of all genes in the gene cluster to be present in that genome. Per outcome group, the frequency of each gene cluster is shown, as are the group-differences. \*These genes were present twice in the genome and count as 1 in for the group total.

**Pneumococcal arginine synthesis genes are associated with disease outcome and infection with pneumococcal serotype 7F in meningitis patients**

In an explorative analysis we evaluated the association between the *argGH* locus and clinical findings in 748 patients with pneumococcal meningitis (Table 4). On admission, patients infected with *argGH* positive pneumococci more often had pneumonia ( $P = .037$ ), and presented with more severe disease, as reflected by a higher proportion of patients in coma ( $P = .002$ ) and focal neurologic deficits ( $P = .042$ ), which resulted in

**Table 3:** Validation of Associations Between Identified Gene Clusters and Outcome in Two Nationwide Cohort Studies (n = 748).

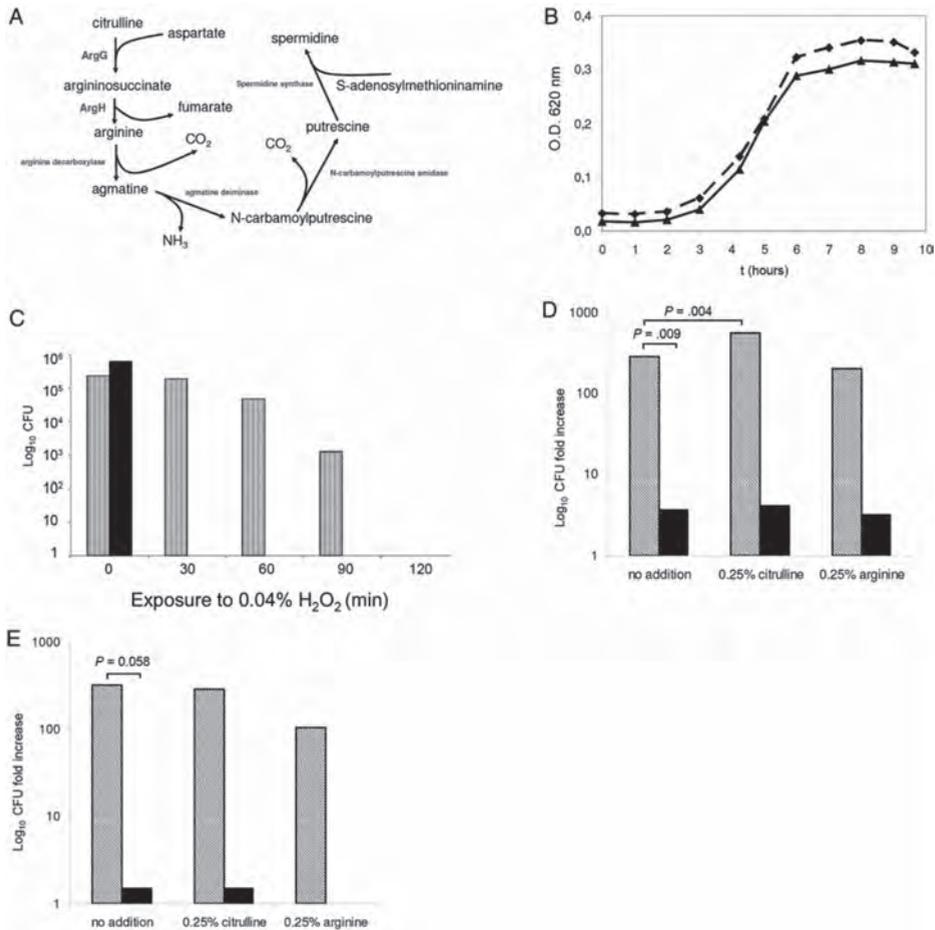
Cluster	Cohort 1			Cohort 2		
	Favorable outcome n = 160 (%)	Unfavorable outcome n = 161 (%)	P	Favorable outcome n = 261 (%)	Unfavorable outcome n = 166 (%)	P
A	100 (63%)	104 (65%)	0.70			
B	44 (28%)	37 (23%)	0.35			
C	23 (14%)	28 (17%)	0.46			
D	47 (29%)	56 (35%)	0.30			
E	75 (47%)	71 (44%)	0.62			
F	56 (35%)	46 (29%)	0.22			
G	<b>32 (20%)</b>	<b>18 (11%)</b>	<b>0.029</b>	35 (13%)	24 (15%)	0.76
H	36 (23%)	35 (22%)	0.87			
I	19 (12%)	29 (18%)	0.12			
J	54 (34%)	45 (28%)	0.26			
K	<b>81 (51%)</b>	<b>64 (40%)</b>	<b>0.050</b>	83 (32%)	50 (30%)	0.72
L	<b>145 (91%)</b>	<b>132 (82%)</b>	<b>0.024</b>	<b>227 (87%)</b>	<b>131 (79%)</b>	<b>0.027</b>
M	73 (46%)	86 (53%)	0.16			
N	25 (16%)	28 (17%)	0.67			

a higher mortality rate ( $P = .003$ ; Figure 3D). In a multivariate regression analysis including pneumonia on admission, admission score on the Glasgow coma scale, and focal neurological deficits, the association of the *argGH* locus with unfavorable outcome was preserved (odds ratio [OR] = 1.62; 95% CI, 1.08–2.42;  $P = .019$ ).

Presence of *argGH* was associated with serotype 7F (60 of 71 [85%] serotype 7F strains were *argGH* positive versus 72 of 677 [11%] non-serotype 7F strains;  $P < .0001$ ; Figure 3E). In a multivariate regression analysis including age, admission score on the Glasgow coma scale, and infection by pneumococcal serotype 7F, the predictive effect of *argGH* on outcome was preserved (OR 1.65; 95% CI, 1.02–2.67;  $P = .040$ ).

## Discussion

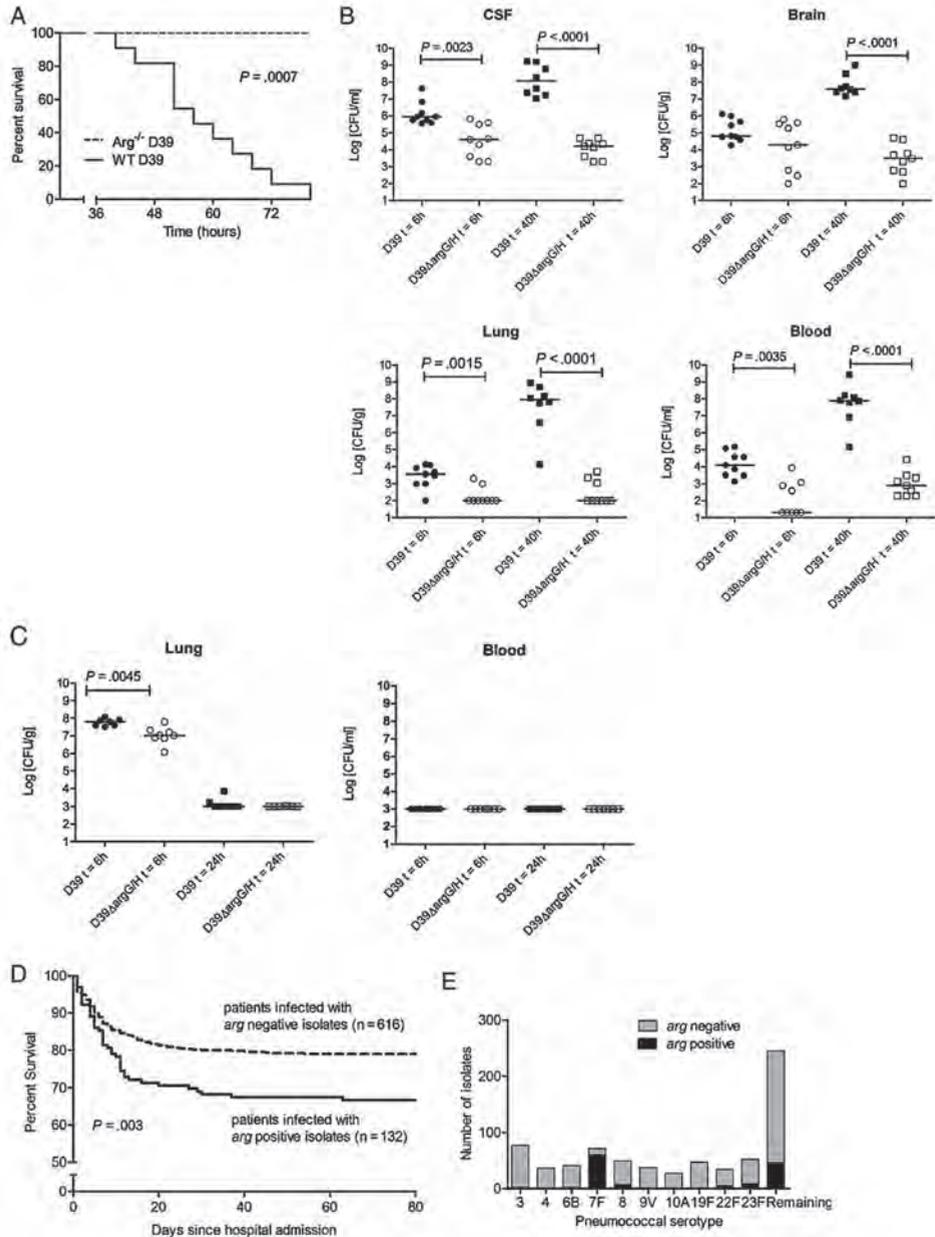
We identified pneumococcal arginine biosynthesis genes associated with outcome in patients with pneumococcal meningitis. Clustered genes were always present on similar genomic loci, implicating regions of plasticity acquired by horizontal gene transfer.<sup>27</sup> Such plasticity regions have been associated with pneumococcal virulence.<sup>21</sup> The region we identified to be associated with disease outcome in our clinical cohort has been identified previously as a plasticity region with a complex history of transposition and integration events.<sup>24</sup> In this region, genes encoding argininosuccinate synthase (*argG*) and argininosuccinate lyase (*argH*) were replaced or truncated by a cluster of genes acquired by horizontal gene transfer. Subsequently, we showed that pneumococci



**Figure 2:** A, Spermidine biosynthesis pathway in *S. pneumoniae*D39. B–E, Bacterial growth of *S. pneumoniae* D39 wild type or arginine biosynthesis genes knock-out strains. *S. pneumoniae* D39 (striped) or *D39ΔargGH* (black) were grown in standard rich laboratory medium THY (B and C), or in blood for 6 hours (D) or in CSF (E). Growth is expressed as the ratio of the number of CFUs of the start inoculum and that after 6 hours growth (CFU fold difference).

harboring these arginine biosynthesis genes show increased resistance to oxidative stress and increased growth in human blood and cerebrospinal fluid. Mouse models of meningitis and pneumonia showed that pneumococcal strains without arginine biosynthesis genes were attenuated in growth and/or cleared from lung, blood and cerebrospinal fluid. The results of our pneumonia model imply that our findings are not limited to pneumococcal meningitis and may well apply to other invasive pneumococcal infections, such as sepsis and pneumonia.

Pneumococci lack a complete arginine biosynthesis pathway. *ArgGH* positive pneumococci convert host's citrulline to arginine, which promotes bacterial growth and contributes to virulence. Arginine biosynthesis genes have been associated with bacterial growth and



**Figure 3:** A, Effect of infection with D39 or D39ΔargGH on survival in mice with pneumococcal meningitis. B, CFUs at 6 and 40 hours in CSF, brain, blood, and lung in mice with pneumococcal meningitis caused by D39 or D39ΔargGH. C, CFUs at 6 and 24 hours in lung and blood in mice with pneumococcal pneumonia caused by D39 or D39ΔargGH. D, Kaplan–Meier survival curve of patients with community-acquired meningitis infected with arg positive (black) and arg negative (striped) *S. pneumoniae*. E, Pneumococcal serotype distribution of arg positive (black) and arg negative (gray) pneumococci.

**Table 4:** Associations of pneumococcal *argGH* locus with clinical characteristics, disease severity and outcome.

Patient Characteristic	<i>S. pneumoniae</i> <i>argGH</i> positive (n = 132)	<i>S. pneumoniae</i> <i>argGH</i> locus truncated or replaced by cluster L (n = 616)	P
Age – year	61 (48-72)	60 (48-70)	0.16
Predisposing conditions			
Otitis/sinusitis	54/132 (41%)	267/615 (43%)	0.60
Pneumonia	33/132 (25%)	106/616 (17%)	0.037
Immunocompromised state <sup>b</sup>	33/132 (25%)	138/615 (22%)	0.53
Signs and symptoms on admission			
Glasgow coma scale score < 8	37/132 (28%)	101/612 (17%)	0.002
Focal neurologic deficits <sup>c</sup>	58/131 (44%)	213/611 (35%)	0.042
Laboratory examination			
CSF white blood cell count/mm <sup>3</sup> <sup>d</sup>	2,899 (416-7,862)	2,500 (575-6,934)	0.97
CSF protein – g/L <sup>e</sup>	5.2 (3.4-7.6)	4.0 (2.5-6.1)	< 0.001
Systemic complications <sup>f</sup>	73/132 (55%)	239/606 (39%)	0.001
Neurologic complications <sup>g</sup>	96/125 (77%)	396/585 (68%)	0.045
Death	46/132 (35%)	138/616 (22%)	0.003
Unfavorable outcome <sup>h</sup>	76/132 (58%)	251/616 (41%)	< 0.001

<sup>a</sup>Data are number/number assessed (%) or median (25th–75th percentile). <sup>b</sup>Defined by the use of immunosuppressive drugs, a history of splenectomy, the presence of diabetes mellitus, alcoholism, and patients infected with HIV. <sup>c</sup>Defined as aphasia, monoparesis, hemiparesis and cranial nerve palsies. <sup>d</sup>CSF leukocyte count was determined in 122 patients infected with *argH* positive and 572 with *argH* negative pneumococci. <sup>e</sup>CSF protein levels were determined in 119 patients infected with *argH* positive and 576 patients with *argH* negative pneumococci. <sup>f</sup>Defined as septic shock, respiratory failure, multiple-organ dysfunction, and cardiac ischemia. <sup>g</sup>Defined as brain herniation, cerebrovascular complications, intractable seizures and withdrawal of care because of poor neurological prognosis. <sup>h</sup>Defined as a score of one to four on the Glasgow outcome scale.

virulence in other pathogens.<sup>24,28</sup> An auxotrophic mutant of *Mycobacterium tuberculosis* defective in L-arginine biosynthesis displayed reduced virulence suggesting that L-arginine availability was restricted in vivo.<sup>24</sup> An *Actinobacillus pleuropneumoniae* transposon mutant of *argG* was attenuated in growth in an experimental pig infection model.<sup>28</sup> Previous in vitro data suggested that arginine uptake is essential for growth of *S. pneumoniae*.<sup>29</sup> In humans, arginine is a substrate of nitric oxide (NO) synthase resulting in the production of citrulline and NO. Citrulline, through the reactions catalyzed by argininosuccinate synthetase and argininosuccinate lyase may cycle back to arginine, constituting an arginine–citrulline cycle.<sup>30</sup> The synthetase ArgG is considered to catalyze the rate-limiting step in NO production and withdrawal of citrulline by pneumococci may reduce NO production, which could be advantageous for bacteria. However, repeated growth experiments with NO synthetase blockers did not influence growth, implying that pneumococcal arginine biosynthesis genes contribute to virulence by influencing bacterial growth only. Arginine is a precursor in the biosynthesis of polyamines, which have been implicated in oxidative stress responses and protection against free radicals.<sup>31</sup> The natural polyamine spermine functions directly as a free radical scavenger<sup>25</sup> and linked to the fitness, survival and pathogenesis of the pneumococcus in host microenvironments.<sup>32</sup>

We showed the  $\Delta argGH$  strain being less resistant to oxidative stress. Polar effects by the knockout mutation on transcription from genes flanking *argGH* was excluded by RT-PCR. Transcription from SP\_0109 (encoding ApbA) was lower in the *argGH* mutant, but a deletion of *apbA* had no effect on growth in low arginine concentration.<sup>29</sup> In addition, in D39 the products of *abpA*, *artP* and *abpB* can substitute for each other.<sup>29</sup> Antibiotics selectively blocking bacterial argininosuccinate synthetase and argininosuccinate lyase could inhibit pneumococcal growth, reducing disease severity.

Presence of arginine biosynthesis genes was associated with pneumococcal serotype 7F, but the association between these genes and clinical outcome even remained robust in a multivariate analysis including pneumococcal serotype. Individual serotypes and major clones of *S. pneumoniae* appear to differ markedly in their potential to cause invasive disease (80–120-fold variation).<sup>9</sup> Serotype 7F has previously been associated with higher invasive disease potential, but with lower case-fatality rates.<sup>33,34</sup> In the Netherlands, 7F has increased during the last decade, being the most frequent serotype in meningitis patients from 2007 onwards.<sup>35</sup> Because of lack of heterogeneity between the OR for invasive disease of different clones of the same serotype, and analysis of isolates of the same genotype, but different serotype, it has been suggested that capsular serotype may be more important than genotype in the ability of pneumococci to cause invasive disease.<sup>9</sup> The effect of *argGH* on disease outcome in patients with pneumococcal meningitis remained robust in a multivariate analysis including serotype.

Our findings are in line with the ‘avirulence hypothesis’ assuming parasites (bacteria, viruses, protozoa and helminths) will evolve to become avirulent.<sup>36</sup> Pneumococci with the *argGH* locus truncated or replaced by cluster L are less fit and virulent. Virulence is considered as a maladaptation of new associations between parasites and hosts.<sup>36</sup> During co-evolution with their host, microorganism may acquire functions that make them more fit in the interaction with their host.<sup>36</sup> Genetic exchange with species sharing the same ecological niche is the main mechanism of evolution of *S. pneumoniae*.<sup>37</sup>

## Supplementary data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/content/suppl/2013/12/13/jit818.DC1>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Declarations

### Author Contributions

J. R. P., A. vd. E. and D. vd. B. conceived and designed the experiments. J. R. P., M. G., B. D. v. S., M. C. B., A. vd. E. and D. vd. B. wrote the manuscript. J. R. P., B. D. v. S. and A. v. K. conducted bioinformatics analyses. M. G., and M. V. S. performed the mouse model experiments, A. H. Z. advised in statistical analyses. J. R. P., M. E. J. and F. B. performed the bacterial genome sequencing. All authors discussed the results and implications, and commented on the manuscript at all stages.

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### Potential conflicts of interest

All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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## Supplementary tables and figures

Supplementary Table 1: Genome assembly strategies.

Bacterial genome	<i>de novo</i> 0 errors	<i>de novo</i> 2 errors	Reference mapping	Cabog
AMCSP01	93	80	136 (NC_008533)	80
AMCSP02	56	61	105 (NC_011072)	65
AMCSP03	75	72	227 (NC_003098)	280
AMCSP04	98	87	128 (NC_003098)	112
AMCSP05	155	148	198 (NC_008533)	240
AMCSP06	83	90	118 (NC_008533)	75
AMCSP07	66	70	108 (NC_008533)	92
AMCSP08	149	110	119 (NC_003098)	81
AMCSP09	144	139	72 (NC_003028)	83
AMCSP10	47	46	98 (NC_011072)	72
AMCSP11	88	86	117 (NC_003098)	70
AMCSP12	87	90	131 (NC_011072)	90
AMCSP13	141	149	85 (NC_003098)	100
AMCSP14	136	133	150 (NC_008533)	95
AMCSP15	64	81	101 (NC_011072)	83
AMCSP16	60	57	105 (NC_008533)	81
AMCSP17	41	49	100 (NC_008533)	102
AMCSP18	104	*	33 (NC_003028)	144
AMCSP19	59	65	113 (NC_008533)	86
AMCSP20	84	82	135 (NC_008533)	92

The number of contigs is shown per assembly strategy: Newbler *de novo* assembler with 0 and 2 errors allowed in the MID calling, a Newbler Reference Mapper assembly (all reference strain GenBank accession numbers producing the least amounts of contigs in brackets: *S. pneumoniae* TIGR4 (NC\_003028), *S. pneumoniae* R6 (NC\_003098), *S. pneumoniae* D39 (NC\_008533), *S. pneumoniae* Hungary 19A-6 (NC\_010380), *S. pneumoniae* CGSP14 (NC\_010582), *S. pneumoniae* G54 (NC\_011072), *S. pneumoniae* Spain23F (NC\_011900) *S. pneumoniae* JJA (NC\_012466), *S. pneumoniae* P1031 (NC\_012467), *S. pneumoniae* 70585 (NC\_012468) and *S. pneumoniae* Taiwan 19F-14 (NC\_012469), and a Cabog assembly. \*Because this strain was sequenced with MID 16 (TCACGTACTA) which has close resemblance to MID 18, no *de novo* assembly with 2 errors allowed in the MID calling procedure was made.

Supplementary Table 2: Available at <http://jid.oxfordjournals.org/content/209/11/1781/suppl/DC1>.

**Supplementary Table 3:** Primers used for cluster determination and constructing bacterial knock-outs.

Primer name	Orientation	DNA sequence	Target Cluster
<b>Cluster determination</b>			
JP_pneu_01	Forward	CCCTGACCCTGCTAGCTCTT	A
JP_pneu_02	Reverse	CTGACGGCCTTACTGATTGG	A
JP_pneu_07	Forward	GATTGGGCTACCCTTTGACA	F
JP_pneu_08	Reverse	TGGATAATCAAGTCCATGCTC	F
JP_pneu_11	Forward	ATGTCGCAAAAAGAGGGTCA	G
JP_pneu_12	Reverse	TTCAACATCTCTGACAAAGTCAA	G
JP_pneu_15	Forward	CTATTGCTGGTTTGGGTGGT	H
JP_pneu_16	Reverse	CAATTGGCATTCCAAAAGAAA	H
JP_pneu_17	Forward	ATGATTTGGAAATCTTAGCGAAA	I
JP_pneu_18	Reverse	TCATTGATCGTTCGCCATTT	I
JP_pneu_21	Forward	TGGCAGCCACTTGTTTATCC	B
JP_pneu_22	Reverse	TGAACGCGAGTAAATGCTTG	B
JP_pneu_23	Forward	TCCATGGCATTACACCTGAA	C
JP_pneu_24	Reverse	GCTTGCTCGTGCATCTGATA	C
JP_pneu_25	Forward	AATCTTGGAGTGGCAACAGG	D
JP_pneu_26	Reverse	TCCTTTTGGAGGACGACTGT	D
JP_pneu_27	Forward	ACAAAGTGGCCGACTACACC	J
JP_pneu_28	Reverse	CAGTGAACCTGCCAATAGCA	J
JP_pneu_31	Forward	TGCTTCGTTTTTGTGGTTAAA	K
JP_pneu_32	Reverse	CTGTGCACCGGAATCAAACA	K
JP_pneu_33	Forward	GAACTCCCTCTCATGCCATC	L
JP_pneu_34	Reverse	AGAGGCAAAAAGCAACGGTTT	L
JP_pneu_35	Forward	CACTACCAGGGAGGGAAACA	M
JP_pneu_36	Reverse	ATCGCAAGTCCCATCACTTC	M
JP_pneu_37	Forward	TTGTGTGGTTCGATTGTGG	E
JP_pneu_38	Reverse	GTTGCAGAATCTCCAAGGA	E
JP_pneu_39	Forward	TCTGAGGGACAACGTCAGC	N
JP_pneu_40	Reverse	CCGTACCATCACGTCTTCCT	N
JP_pneu_43	Forward	TGGGTAGAGCGCTTTGGTGCG	<i>argH</i>
JP_pneu_44	Reverse	GCCATAAACTCTGCCAGTCTTTCC	<i>argH</i>
JP_pneu_45	Forward	TCTGCCTTGAGCCGCCCTCT	<i>argG</i>
JP_pneu_48	Reverse	TTTGCAGTCCCATTGACAAC	<i>argG</i>
<b>Constructing bacterial knock-outs</b>			
KSPNArg2061617F8		GGCTGGAATTGAAGCC	
KSPNArg2061617R6		CGAAACCGCCACAATAGGATCCAAAATTAC	
KSPNArg2061617F7		ATTAAAGGGCCCATGATGCATATGAGTCG	
KSPNArg2061617R9_1		CGACTTGGTCAAACC	
KSPNArg2061617F1		GCTATCGCAACAGAACTAGG	
KSjanuskanaR1		AAAGATTATATCACATTATCCATT	

Primer name	Orientation	DNA sequence	Target Cluster
<b>RT PCR</b>			
KSABCF		GAACGGAGCAAGGTGTTG	
KSABCR		AAATAACGGCATCCACTTGC	
KSarghypF		AGATCGTGGTGGGTCTTGAG	
KsarghypR		GGCAAGACAACCCAAAATA	
Gki up		GGCATTGGAATGGGATCACC	
Gki dn		TCTCCCGCAGCTGACAC	

For each gene (rows), the frequency per genome is scored (columns).



# 7

## Cerebrospinal fluid inflammatory mediators and outcome in patients with pneumococcal meningitis

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*Submitted for publication*

## Abstract

**Background.** The severity of the cerebrospinal fluid (CSF) inflammatory response in pneumococcal meningitis has been associated with brain damage and unfavorable outcome.

**Methods.** We analyzed 88 inflammatory mediators in the CSF of adult patients with pneumococcal meningitis included in a prospective cohort study (MeninGene). We compared CSF levels of inflammatory mediators between patients with favorable and unfavorable outcome, defined as a Glasgow Outcome Scale (GOS) score below 5. We used CSF of patients diagnosed with thunderclap headache as negative controls.

**Results.** CSF was available from 271 patients with pneumococcal meningitis and 19 controls. Unfavorable outcome was reported in 109 patients (40%), and 35 patients (13%) died. After Bonferroni correction for multiple testing ( $p < 0.0057$ ) 59 mediators were significantly elevated in the CSF of pneumococcal meningitis patients compared to negative controls. CSF levels of sICAM-1, MMP-1, MMP-3, IL-1RA, IL-10, CXCL5 and CCL7 were associated with unfavorable outcome. High CSF levels of sICAM-1 and MMP-1 were associated with mortality.

**Conclusion.** We identified an adhesion molecule (sICAM-1), two matrix metalloproteinases (MMP-1 and MMP-3), two chemokines (CXCL5 and CCL7) and two anti-inflammatory cytokines (IL-10 and IL-1RA) to be associated with unfavorable outcome in patients with pneumococcal meningitis, which suggests they might be involved in the pathophysiology of this disease.

## Introduction

Bacterial meningitis is an infection of the membranes surrounding the brain and is associated with high mortality and morbidity rates.<sup>1,2</sup> *Streptococcus pneumoniae* is the most common causative agent of bacterial meningitis in adults, causing 70% of cases.<sup>3,4</sup> The reported incidence of pneumococcal meningitis has lowered to 0.8 per 100,000 persons per year, since routine vaccination of children with the seven- and ten-valent pneumococcal conjugate vaccines was introduced in the Netherlands in 2006 and 2011.<sup>4</sup> In low-income countries the incidence remains substantially higher.<sup>5</sup> In high-income countries, the mortality rate among patients with pneumococcal meningitis in adults is 18%, and up to half of surviving patients suffer from neurologic sequelae including cognitive impairment.<sup>3,6</sup>

Experimental studies showed that brain damage and adverse outcome results mainly from the central nervous system (CNS) inflammatory response caused by the interaction between host and pathogen.<sup>7,8,9,10</sup> Several inflammatory mediators have been found elevated in the CSF in patients with pneumococcal meningitis.<sup>11,12</sup> Comprehensive prospective studies analyzing the association between large number of different cytokines, chemokines or endothelial growth factors with outcome are not available. In 2006, we started a prospective cohort study to identify and characterize host genetic traits and bacterial genetic factors controlling occurrence and outcome of bacterial meningitis (MeninGene).<sup>4,13-15</sup> In this study we also collected CSF of the diagnostic lumbar puncture of patients with bacterial meningitis. To gain more insight in the pathophysiology of pneumococcal meningitis to identify potential new targets for adjuvant treatments, we performed an explorative study analyzing 88 different inflammatory mediators in the CSF of patients with pneumococcal meningitis.

## Material and methods

### Dutch bacterial meningitis cohort

We conducted a prospective nationwide cohort study in the Netherlands including adult patients (over 16 years of age) with community acquired bacterial meningitis confirmed by positive CSF cultures. Patients were identified by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) from March 2006 to August 2010, which receives bacterial strains from approximately 85% of all patients with bacterial meningitis in the Netherlands. The treating physician was contacted, and informed consent was obtained from all participating patients or their legally authorized representatives.

Patients with hospital-acquired bacterial meningitis, those with a neurosurgical device or negative CSF cultures were excluded. Clinical information on the disease course was collected in a secured online case-record form. Outcome was assessed at discharge by the treating physician using the Glasgow Outcome Scale (GOS), a 5 point scale in which a

score of 5 indicates mild or no disability (the patient is able to return to work or school), a score of 4 indicates moderate disability (able to live independently), a score of 3 indicates severe disability (unable to live independently), a score of 2 represents a vegetative state and a score of 1 death. A score of 1-4 was defined as unfavorable outcome, a score of 5 as favorable outcome.

When leftover CSF from the first diagnostic lumbar puncture was available, it was spun down and supernatant was locally stored at -80 degrees for study purposes. Participating centers were regularly visited by the investigators to collect the CSF samples and transport them to the Academic Medical Center Amsterdam on dry ice for further storage until measurements were performed. Samples were thawed, spun down and the supernatant was aliquoted in 30  $\mu$ l volumes. CSF samples from 19 patients with thunderclap headache in whom subarachnoidal hemorrhage was excluded by CSF examination, served as negative controls. All these CSF samples had normal leukocyte count, protein and glucose levels. The study was approved by the Medical Ethics Committee (MEC) of the Academic Medical Center Amsterdam.

### **Analysis of inflammatory mediators**

We measured 88 inflammatory mediators in CSF of 271 patients with pneumococcal meningitis and 19 negative controls. Analytes included CCL1 to CCL5, CCL7, CCL8, CCL13, CCL14a, CCL15, CCL17, CCL19 to CCL22, CCL24, CCL26, CCL27, CXCL1, CXCL5 to CXCL7, CXCL9 to CXCL11, CXCL12a and b, CXCL13, XCL1, CX3CL1, interleukin (IL)-1- $\alpha$ , IL-1- $\beta$ , interleukin-1 receptor antagonist (IL-1RA), IL-2 to IL-11, IL-12p40, IL12p70, IL-13, IL-15 to IL-18, IL-20, IL-21, IL-23, IL-28A, IL-29, IL-33, interferon (IFN)- $\alpha$ 2, IFN- $\gamma$ , leukemia inhibitory factor (LIF), thyroid peroxidase (TPO), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), stem cell factor (SCF), thymic stromal lymphopoietin (TSLP), macrophage colony stimulating factor (M-CSF), epidermal growth factor (EGF), eotaxin, fibroblast growth factor (FGF)-2, FMS-related tyrosine kinase (Flt)-3 ligand, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), platelet derived growth factor (PDGF)-AA, PDGF-AB/BB, soluble cluster of differentiation 40 ligand (sCD40L), soluble interleukin-2 receptor alpha (sIL-2Ra; sCD25), transforming growth factor alpha (TGF- $\alpha$ ), tumor necrosis factor alpha (TNF)- $\alpha$ , TNF- $\beta$ , vascular endothelial growth factor (VEGF), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule (sICAM)-1, matrix metalloproteinase (MMP)-1 to MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, MMP-13 and macrophage migration inhibitory factor (MIF). This selection was based on the availability of the combination of specific cytokines and chemokines of our interest included in Luminex assays by the manufacturer. All analytes were measured with Luminex<sup>®</sup> xMAP<sup>®</sup> technology using Milliplex<sup>®</sup> map multiplex assay's (Millipore Billerica, MA, USA). CSF was diluted 1:50 in assay buffer provided with the Multiplex assay and inflammatory mediators were measured according to the manufacturer's instructions. Plates were read with the Bio-Plex<sup>®</sup> 200 system array reader.

Bio-Plex Manager™ software was used for data acquisition and analysis (Bio-Rad Hercules, CA, USA). All concentrations are given in pg/ml.

### Statistics

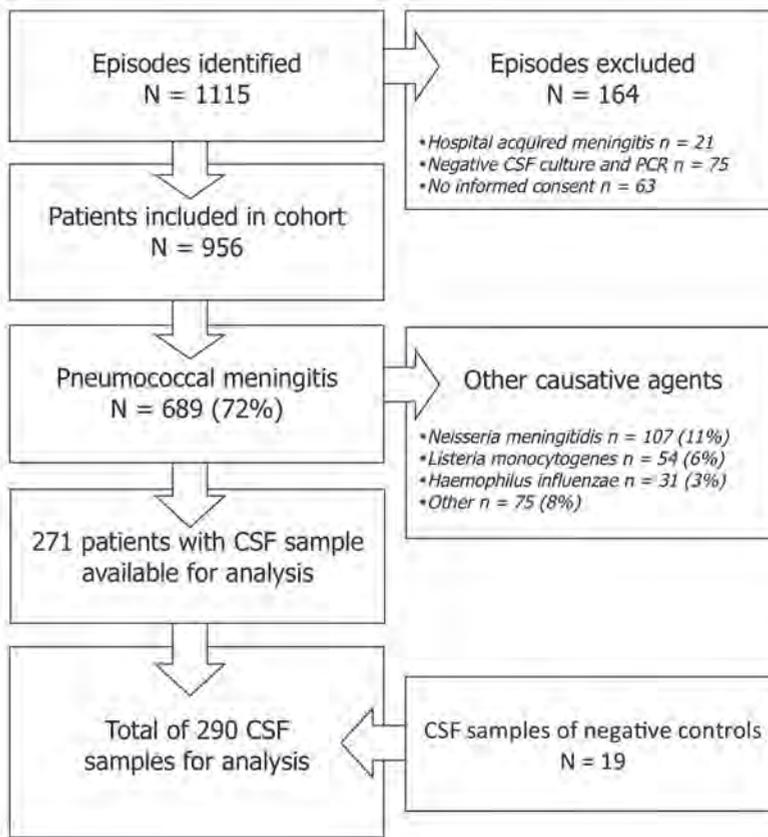
Data below the lower limit of quantification (LLOQ) of the assay were imputed with a random value between zero and the individual LLOQ of the analyte. Data above the upper limit of quantification (ULOQ) of the assay were assigned the value of the ULOQ of the individual analyte. Levels of inflammatory mediators were compared between groups with a Mann-Whitney U test. Continuous data are presented as median with interquartile range. All statistical tests were 2-tailed, and a Bonferroni-corrected P-value  $< 0.00057$  ( $0.05/88$ ) was considered significant. Spearman's correlation test was used to identify correlations between levels of immune mediators in the CSF. Analyses were done using IBM SPSS statistics version 22.0. Statistical correction for batch effect was performed. We conducted a principal component analysis (PCA) and linear determinant analysis (LDA) after log transformation and standardizing the data, to identify clusters of immune mediators correlated to outcome, using R statistics version 3.2.3.<sup>16</sup> We conducted a supervised pathway analysis using QIAGEN's Ingenuity Pathway Analysis (IPA).

## Results

### Study cohort

We identified 1115 episodes of bacterial meningitis in 1100 patients with bacterial meningitis through the NRLBM between January 2006 and November 2010. Of 1115 episodes, in 75 cases CSF culture was negative, 21 cases had hospital-acquired meningitis and in 63 cases no informed consent was obtained. Of 956 cases included in the study 689 (72%) had *S. pneumoniae* as a causative agent. CSF from the diagnostic lumbar puncture was available for analysis in 271 of 689 patients (39%) (figure 1). Patients with CSF available, had similar baseline characteristics as patients without CSF available, except for a lower mortality rate (22% versus 13%;  $p = 0.002$ ; table 1).

Median age of patients with available CSF samples was 61 years, and 128 patients (47%) were male, 126 (48%) had symptoms less than 24 hours and 107 (66%) had a predisposing condition such as sinusitis, otitis, pneumonia, or an immunocompromised state. At presentation 200 patients (80%) had fever, 218 (80%) had an altered mental status, 58 (23%) had focal neurological deficits and 57 (21%) showed signs of septic shock. CSF of patients with pneumococcal meningitis showed an elevated leukocyte count of 2,560 cells/mm<sup>3</sup> (IQR 514 – 6,655), high protein content of 4.0 g/L (IQR 2.5 – 6.0), and low glucose CSF-blood ratio 0.023 (IQR  $<0.001$  – 0.233). Outcome was unfavorable in 109 patients (40%) and 35 patients (13%) died (table 1).



**Figure 1.** Flow chart of patients included in the MeninGene study and negative controls.

### CSF inflammatory mediators in patients with pneumococcal meningitis

Inflammatory mediators were successfully measured in 98-100% of samples. Invalid measurements were due to technical aspects such as too small amounts of CSF available for multiple assays or clogging of the luminex beads. Of the inflammatory mediators measured 59 were elevated in the CSF of patients with pneumococcal meningitis compared to negative controls (supplementary table 1). The ten most significantly elevated analytes included MMP-9 (median 497,193 versus 109 pg/ml; p-value  $2.69 \times 10^{-21}$ ), CCL2 (median 99,802 versus 465 pg/ml; p-value  $1.49 \times 10^{-16}$ ), IL-6 (median 78,636 versus 13 pg/ml; p-value  $4.97 \times 10^{-13}$ ), G-CSF (median 16,372 versus 55 pg/ml; p-value  $8.59 \times 10^{-13}$ ), CXCL1 (median 21,574 versus 55 pg/ml; p-value  $1.30 \times 10^{-12}$ ), CXCL8 (median 53,241 versus 27 pg/ml; p-value  $1.41 \times 10^{-12}$ ), sICAM-1 (median 60,375 versus 2,292 pg/ml; p-value  $1.75 \times 10^{-12}$ ), IL-10 (median 1,868 versus 4 pg/ml; p-value  $2.14 \times 10^{-12}$ ), IL-1 $\beta$  (median 1,875 versus 4 pg/ml; p-value  $2.21 \times 10^{-12}$ ) and IL-1RA (median 4,030 versus 22 pg/ml; p-value  $3.18 \times 10^{-12}$ ).

**Table 1.** Baseline characteristics of patients with CSF sample compared to patients without a CSF sample available.

Characteristic	Patients with CSF sample (N = 271)	Patients without CSF sample (N = 418)	P-value
<i>General</i>			
Age	61 (49 – 70)	61 (50 – 69)	0.89
Male sex	128/271 (47%)	209/418 (50%)	0.48
Duration of symptoms < 24 hours	126/263 (48%)	198/405 (49%)	0.81
<i>Predisposing conditions</i>			
Otitis	90/263 (34%)	139/397 (35%)	0.84
Sinusitis	37/260 (14%)	53/391 (14%)	0.50
Pneumonia	25/262 (10%)	48/402 (12%)	0.34
Immunocompromised state <sup>a</sup>	82/271 (30%)	105/418 (25%)	0.14
<i>Presenting symptoms</i>			
Temperature > 38 °C	200/249 (80%)	322/392 (82%)	0.56
Focal neurological deficits	58/255 (23%)	96/393 (25%)	0.62
Signs of septic shock <sup>b</sup>	57/267 (21%)	102/409 (24%)	0.28
Altered mental status (GCS < 14)	218/271 (80%)	329/418 (79%)	0.58
<i>Cerebrospinal fluid</i>			
White blood cell count (/mm <sup>3</sup> )	2560 (514 – 6655)	2800 (549 – 7339)	0.40
White blood cell count <1000/mm <sup>3</sup>		124/387 (32%)	0.58
Protein (g/L)	4.0 (2.5 – 6.0)	4.3 (2.5 – 6.3)	0.24
CSF blood glucose ratio	0.023 (0.00 – 0.23)	0.016 (0.00 – 0.15)	0.05
<i>Clinical course</i>			
Neurologic complications <sup>c</sup>	163/207 (79%)	262/324 (81%)	0.55
Systemic complications <sup>d</sup>	117/266 (44%)	181/409 (44%)	0.95
Unfavorable outcome	109/271 (40%)	180/418 (43%)	0.46
Death	35/271 (13%)	92/418 (22%)	0.002

Data are number/number evaluated (%), and median (interquartile range). <sup>a</sup>Immunocompromised state was defined as the use of immunosuppressive drugs, the presence of diabetes mellitus or alcoholism, and human immunodeficiency virus (HIV) infection. <sup>b</sup>Signs of septic shock were defined as a systolic blood pressure <90 mmHg, a diastolic blood pressure <60 mmHg and/or heart rate >120/min. <sup>c</sup>Neurologic complications were defined as an altered mental status, focal neurologic deficits, hydrocephalus, seizures, cerebrovascular events (infarction, hemorrhage, cerebral venous sinus thrombosis), cerebral abscess or empyema during admission. <sup>d</sup>Systemic complications were defined as septic shock (diastolic blood pressure <60 mmHg), respiratory failure and need for mechanical ventilation during admission.

### CSF inflammatory mediators associated with outcome in pneumococcal meningitis

After Bonferroni correction, unfavorable outcome in pneumococcal meningitis was associated with high CSF levels of MMP-1 (median 4,441 versus 1,530 pg/ml; p-value  $4.95 \times 10^{-10}$ ), sICAM-1 (median 78,580 versus 50,530 pg/ml; p-value  $9.36 \times 10^{-07}$ ), IL-1RA (median 6,122 versus 2,879 pg/ml; p-value  $9.36 \times 10^{-06}$ ), CXCL5 (median 2,410 versus 681 pg/ml; p-value  $1.41 \times 10^{-05}$ ), IL-10 (median 2,787 versus 1,204 pg/ml; p-value  $2.15 \times 10^{-05}$ ), MMP-3 (median 74,491 versus 21,286 pg/ml; p-value  $9.42 \times 10^{-05}$ ) and CCL7 (median 1,694 versus 920 pg/ml; p-value  $3.20 \times 10^{-04}$ ) (table 2). All of these analytes were elevated compared to negative controls except CXCL5 (median 854 pg/ml versus 577 pg/ml; p-value  $8.89 \times 10^{-03}$ ). CSF levels of CXCL5 were elevated above the LLOQ in only 40% of patients. Results of all inflammatory mediators in patients with favorable versus unfavorable outcome are listed in supplementary table 2.

Mortality in pneumococcal meningitis was associated with high CSF levels of sICAM-1 (median 125,860 versus 55,230 pg/ml; p-value  $1.49 \times 10^{-06}$ ) and MMP-1 (median 8,904 versus 2,101 pg/ml; p-value  $1.11 \times 10^{-05}$ ) (table 2). Both sICAM-1 (71,875 pg/ml versus 52,810 pg/ml; p-value  $1.19 \times 10^{-04}$ ) and MMP-1 (3483 pg/ml versus 1612 pg/ml; p-value  $1.40 \times 10^{-05}$ ) levels were higher in patients with systemic complications (defined as septic shock (diastolic blood pressure <60 mmHg), respiratory failure and need for mechanical ventilation during admission). We did not identify CSF inflammatory mediators that were associated with neurologic complications, hearing loss or cognitive impairment (data not shown). Correction for batch effect did not influence results (data not shown).

IL-1RA blocks the binding of IL-1 $\alpha$  and IL-1 $\beta$  to the IL-1 receptor. Previous human sepsis studies reported that a 100-1000 fold increase of IL-1RA levels compared to IL-1 $\beta$  levels are needed to antagonize the effect of IL-1 $\beta$ .<sup>17,18</sup> In our population IL-1RA/IL-1 $\beta$  ratio was higher in patients with unfavorable outcome compared to patients with favorable outcome (ratio 2.52 versus 1.65; p-value 0.018) and in deceased compared to patients that survived (ratio 3.59 versus 1.73; p-value 0.028). However the IL-1RA/IL-1 $\beta$  ratio was substantially lower than the previously reported 100-fold relative increase of IL-1RA needed to antagonize the effect of IL-1 $\beta$ .

**Table 2.** CSF concentrations of inflammatory mediators in patients with pneumococcal meningitis.

OUTCOME			
Analyte	Favorable (n = 162)	Unfavorable (n = 109)	p-value
MMP-1	1530 (520 - 3,506)	4,441 (1,822 - 9,578)	$4.95 \times 10^{-10}$
sICAM-1	50,530 (29,110 - 78,095)	78,580 (47,375 - 135,260)	$9.36 \times 10^{-07}$
IL-1RA	2,879 (905 - 6,777)	6,122 (3,325 - 11,492)	$9.36 \times 10^{-06}$
CXCL5	681 (318 - 1,492)	2,410 (659 - 4,536)	$1.41 \times 10^{-05}$
IL-10	1,204 (464 - 3,270)	2,787 (1,308 - 5,899)	$2.15 \times 10^{-05}$
MMP-3	21,286 (6,487 - 77,267)	74,491 (16,062 - 159,301)	$9.43 \times 10^{-05}$
CCL7	920 (377 - 2,042)	1,694 (726 - 3,955)	$3.20 \times 10^{-04}$
SURVIVAL			
Analyte	Survived (n = 236)	Died (n = 35)	p-value
sICAM-1	55,230 (34,720 - 84,610)	125,860 (69,300 - 183,480)	$1.49 \times 10^{-06}$
MMP-1	2,101 (826 - 4,601)	8,904 (2,605 - 12,096)	$1.11 \times 10^{-05}$

CSF concentrations (pg/ml) of inflammatory mediators in patients with pneumococcal meningitis with favorable (GOS 5) versus unfavorable outcome (GOS  $\leq 4$ ) and patients that survived versus patients that died. Data are given as median with interquartile range.

### Correlations with patient characteristics and other inflammatory mediators

Inflammatory mediators in CSF were not substantially correlated to blood or CSF leukocyte count (data not shown). We found moderate to strong positive correlations between the seven inflammatory mediators associated with outcome and CSF protein content, and moderate negative correlations between these inflammatory mediators and CSF blood-glucose ratio (table 3). The seven inflammatory mediators associated with outcome, were not associated with age or GCS on admission (data not shown). Among the inflammatory

mediators found to be associated with unfavorable outcome, we found a strong correlation between IL-1RA and IL-10 levels ( $\rho$  0.80; p-value  $4.48 \times 10^{-62}$ ).

### Cluster- and pathway analysis

We performed a PCA on all patients to find unsupervised clustering of inflammatory mediators. There was no separation based on levels of CSF immune mediators indicating a favorable or unfavorable outcome (supplementary figure 1). We used a supervised approach and conducted a LDA and pathway analysis using Ingenuity Pathway Analysis (IPA). The LDA showed that the best separation between favorable and unfavorable outcome were the strongest significantly associated results in the univariate analysis with unfavorable outcome (MMP-1, sICAM-1, IL-1RA, CXCL5, IL-10, MMP-3 and CCL7), and in addition CCL5, MIF, MMP-10, TGF- $\alpha$ , TRAIL, IL-12p40 and MMP-12. We performed a second supervised pathway analysis using IPA. The annotations found included 'recruitment of phagocytes' and 'recruitment of neutrophils', but gave no new insights in the dataset.

**Table 3.** Correlations between CSF inflammatory mediators and CSF total protein and CSF blood-glucose ratio.

Analyte	CSF total protein		CSF-blood glucose count	
	$\rho$	p-value	$\rho$	p-value
MMP-1	0.58	$8.08 \times 10^{-25}$	-0.59	$3.64 \times 10^{-22}$
sICAM-1	0.65	$1.21 \times 10^{-31}$	-0.59	$6.13 \times 10^{-22}$
IL-1RA	0.59	$1.44 \times 10^{-25}$	-0.64	$2.32 \times 10^{-27}$
CXCL5	0.37	$1.97 \times 10^{-08}$	-0.38	$7.79 \times 10^{-08}$
IL-10	0.53	$2.81 \times 10^{-20}$	-0.59	$1.07 \times 10^{-22}$
MMP-3	0.54	$1.87 \times 10^{-21}$	-0.50	$7.11 \times 10^{-16}$
CCL7	0.54	$4.24 \times 10^{-21}$	-0.58	$8.34 \times 10^{-22}$

The Spearman's Rank Correlation Coefficients ( $\rho$ ) were calculated with a Spearman's test. A p-value below 0.00057 was considered significant.

## Discussion

We identified seven CSF inflammatory mediators to be associated with unfavorable outcome in patients with pneumococcal meningitis: an adhesion molecule (sICAM-1), two matrix metalloproteinases (MMP-1 and MMP-3), two chemokines (CXCL5 and CCL7) and two anti-inflammatory cytokines (IL-10 and IL-1RA). High levels of CSF inflammatory mediator levels correlated with markers of severity of the inflammation, such as low CSF blood-glucose ratio and high CSF protein content (except for CXCL5), but were independent of CSF leukocyte count or levels of disease severity, as reflected by the weak correlation with GCS score on admission.

CSF sICAM-1 levels were associated with unfavorable outcome and death in pneumococcal meningitis. Our data suggest that the role of sICAM-1 in pneumococcal meningitis is independent of leukocyte infiltration. ICAM-1 is a transmembrane protein involved in leukocyte diapedesis.<sup>19</sup> In addition to membrane bound ICAM-1, activated

cells are able to produce a soluble form of ICAM-1 which is secreted.<sup>20</sup> The function of circulating sICAM-1 is yet unknown. Soluble ICAM-1 can bind to the receptor lymphocyte function-associated antigen (LFA)-1, suggesting a role in inhibiting the binding of leukocytes to endothelial cells, but it also may also be released as a result of tissue damage, thereby promoting inflammation.<sup>20</sup> In our study we found no correlation between CSF sICAM-1 concentration and CSF leukocyte count. The lack of association between sICAM-1 and leukocyte count is in line with a previous study showing a similar degree of neutrophil infiltration in the lung in ICAM-1 deficient mice compared to wild type mice in a pneumococcal pneumonia model.<sup>21</sup>

We identified two matrix metalloproteinases (MMP-1 and MMP-3) to be associated with outcome. MMP-1 is a collagenase whose production can be induced by several cytokines such as IL-1 $\beta$ , IL-31 or activation of Toll-like receptor (TLR)2.<sup>22,23,24</sup> Substrates of MMP-1 include multiple extracellular components, but also several inflammatory components including L-selectin, pro-TNF, IL-1 $\beta$ , MMP-2, MMP-9 and several chemokines of which some are cleaved into receptor antagonists and may exert an anti-inflammatory effect.<sup>25</sup> Not much is known about the role of MMP-1 in pneumococcal disease. A selective MMP blocker of MMP-1, -8 and -13 was used in a pneumococcal meningitis model in rats, showing reduced mortality, CSF TNF- $\alpha$  and IL-1 $\beta$  concentrations and less hippocampal apoptosis and cortical necrosis on brain pathology.<sup>26</sup> To what extent blocking of MMP-1 is responsible for these effects is unknown. MMP-3 degrades multiple extracellular matrix components and can activate several other MMP's such as MMP-1, MMP-7 and MMP-9.<sup>25</sup> The function of MMP-3 and its role in invasive pneumococcal disease is unclear. In a rat model of pneumococcal meningitis MMP-3 mRNA was upregulated in brain tissue.<sup>27</sup> Furthermore, a role for MMP-3 in the activation of MMP-1 has been described,<sup>25</sup> which was also associated with unfavorable outcome in our study.

IL-1RA is a receptor antagonist that prevents binding of both IL-1 $\alpha$  and IL-1 $\beta$  to the IL-1 receptor, resulting in a reduced release of IL-6, TNF and IL-1 by monocytes, and subsequent reduced recruitment of neutrophils.<sup>18</sup> IL-1RA was previously shown to be present in the CSF of patients with bacterial meningitis and high levels were associated with fatal disease<sup>28</sup>. From experimental studies we know that IL-1RA plays a role in the prevention of host tissue damage during pneumococcal infection.<sup>29,30</sup> In human sepsis studies and in vitro, a 100-1000 fold increase of IL-1RA levels compared to IL-1 $\beta$  levels was shown to fully antagonize the effect of IL-1 $\beta$ .<sup>17,18</sup> In our study we found higher IL-1RA/IL-1 $\beta$  ratio in patients with unfavorable outcome compared to those with favorable outcome. However, IL-1RA/IL-1 $\beta$  ratios were well below the levels needed to antagonize IL-1 $\beta$ . The increased IL-1RA levels in patients with an unfavorable outcome may represent a failing attempt to control the inflammation, as these levels appear to be insufficient to fully antagonize the effect of IL-1 $\beta$ .

IL-10 is an anti-inflammatory cytokine. In experimental studies it was shown that IL-10 reduces the expression of pro-inflammatory cytokines and the recruitment of neutrophils, leading to less collateral tissue damage but also a less efficient clearance of

bacteria.<sup>31</sup> In children with bacterial meningitis CSF IL-10 levels were associated with high bacterial loads.<sup>32</sup> We found that high CSF IL-10 levels were associated with unfavorable outcome, and that higher levels were strongly associated with co-existing CSF levels of the anti-inflammatory IL-1RA. Similar to IL1-RA the high levels of IL-10 could indicate a failing compensatory mechanism to control the inflammatory response, or high IL-10 levels may lead to over suppression of the immune response and thereby decreased bacterial clearance and adverse outcome.

We found two chemokines, CCL7 and CXCL5, to be elevated in the CSF of pneumococcal meningitis patients with unfavorable outcome. CCL7 is a monocyte and neutrophil chemo-attractant.<sup>33,34</sup> CCL7 is modulated by MMP-2 and MMP-12, and both are able to cleave CCL7 into a receptor antagonist, thereby inhibiting chemotaxis.<sup>34,35</sup> In pneumococcal disease, CCL7 is upregulated in response to pneumolysin and antibodies against CCL7 reduced neutrophil recruitment in experimental pneumococcal pneumonia.<sup>36,37</sup> CXCL5 is a neutrophil attractant and activator, which has shown to be produced by a human alveolar epithelial cell line in response to choline-binding protein A (CbpA), together with ICAM-1.<sup>38,39</sup> In previous reports CXCL5 was demonstrated in the CSF of patients with bacterial meningitis where it exerted a chemotactic activity towards neutrophils.<sup>40</sup> In our study neither CCL7 nor CXCL5 levels were associated with CSF leukocyte count, which could suggest a different function for these chemokines in the pathophysiology of pneumococcal meningitis.

Our study has several limitations. First, as we only included culture- or PCR proven bacterial meningitis we have a selected group of patients. Negative cerebrospinal fluid cultures occur in 11 to 20% of patients with bacterial meningitis.<sup>41</sup> Furthermore, patients of whom CSF was available showed a lower mortality rate than the patients of whom CSF was not obtained for this study. This would probably lead to an underestimation of the associations found rather than an overestimation. The second limitation is the potentially different handling of CSF samples. Our study was a multicenter study, and although we provided and agreed a laboratory protocol with all participating hospitals there might have been differences in handling of the CSF samples in the local hospitals before storage at -80 degrees. Also, the time between CSF collection, storage and analysis of the inflammatory mediators varied between samples. This may specifically have implications for the comparisons between CSF samples of patients with high leukocyte contents and CSF of negative controls with relatively few leukocytes. In the analyses where patients with favorable versus unfavorable outcome were compared, differences in sample handling are expected to have occurred at random in both groups, and therefore it is less likely that they have influenced the results. MMP-9, CCL2 and IL-6 levels were elevated above the ULOQ in a large percentage of samples, an association with unfavorable outcome could therefore be missed. Finally, we cannot draw conclusions from this study about the causality of the increase in inflammatory mediators in patients with an unfavorable outcome. The elevation of CSF inflammatory mediators could for instance be due to higher bacterial loads or uncontrolled inflammatory response. It is unknown whether the inflammatory

mediators measured are in active or inactive state, or are cleaved in agonist or antagonist variant where applicable. Further research is needed to address these questions.

In conclusion, we identified CSF levels of an adhesion molecule (sICAM-1), two matrix metalloproteinases (MMP-1 and MMP-3), two anti-inflammatory cytokines (IL-10 and IL-1RA) and two chemokines (CXCL5 and CCL7) to be associated with unfavorable outcome in patients with pneumococcal meningitis. These results provide new leads for risk assessment for patients with pneumococcal meningitis and may point to the direction of new specific adjuvant treatments aiming at improved outcome of pneumococcal meningitis.

## **Declarations**

### **Author Contributions**

The work presented here was carried out in collaboration between all authors. Multiplex assays were carried out by MG and MVS. AZ and BF contributed to the statistical analysis. The manuscript was drafted by MG and discussed and edited by MCB, AE and DB. All authors have read and approved the final version of the manuscript.

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### **Potential conflicts of interest**

The authors have no conflicts of interest to report.

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## Supplementary tables and figures

**Supplementary table 1:** CSF inflammatory mediator levels of patients with pneumococcal meningitis and negative controls.

Analyte	<i>S. pneumoniae</i> meningitis			Negative controls		P-value
	%< LLOQ	%> ULOQ	N <sup>a</sup>	Median concentration	Median concentration	
MMP-9	0	81	268	497,193 (497,193 - 497,193)	109 (59 - 160)	2.69x10 <sup>-21</sup>
CCL2	1	76	269	99,802 (65,741 - 99,802)	465 (374 - 582)	1.49x10 <sup>-16</sup>
IL-6	1	33	270	78,635 (43,092 - 99,232)	13 (9 - 34)	4.97x10 <sup>-13</sup>
GCSF	2	0	269	16,372 (6,463 - 31,464)	55 (36 - 96)	8.59x10 <sup>-13</sup>
CXCL1	3	1	270	21,574 (4,973 - 40,859)	55 (21 - 88)	1.30x10 <sup>-12</sup>
CXCL8	1	0	270	53,241 (18,150 - 116,520)	27 (16 - 39)	1.41x10 <sup>-12</sup>
sICAM-1	1	0	266	60,375 (37,535 - 93,413)	2,292 (995 - 5,090)	1.75x10 <sup>-12</sup>
IL-10	2	0	270	1,868 (565 - 3,982)	4 (2 - 6)	2.14x10 <sup>-12</sup>
IL-1β	2	0	270	1,875 (292 - 5,968)	4 (2 - 6)	2.21x10 <sup>-12</sup>
IL-1RA	3	0	270	4,030 (1,318 - 8,925)	22 (8 - 31)	3.18x10 <sup>-12</sup>
IFN-α2	4	0	265	1,182 (626 - 1,763)	67 (7 - 125)	4.56x10 <sup>-12</sup>
IFN-γ	3	0	268	1,260 (503 - 4,271)	18 (9 - 32)	5.76x10 <sup>-12</sup>
TNF- α	8	0	269	3,488 (631 - 11,467)	50 (32 - 61)	1.72x10 <sup>-11</sup>
IL-17	6	0	270	145 (64 - 269)	5 (3 - 5)	2.25x10 <sup>-11</sup>
CCL4	9	0	270	16,440 (4,621 - 39,995)	574 (309 - 811)	2.63x10 <sup>-11</sup>
CCL20	4	0	226	6,790 (1,957 - 17,105)	23 (10 - 28)	3.22x10 <sup>-11</sup>
GMCSF	6	0	270	895 (313 - 1,768)	18 (9 - 31)	3.26x10 <sup>-11</sup>
CXCL10	4	16	270	129,928 (60,277 - 317,858)	425 (115 - 679)	3.86x10 <sup>-11</sup>
IL-1α	7	0	270	482 (203 - 1,302)	18 (11 - 21)	4.34x10 <sup>-11</sup>
IL-12p70	9	0	269	136 (77 - 205)	19 (13 - 29)	7.02x10 <sup>-11</sup>
MMP-3	8	3	270	37,537 (9,408 - 119,713)	1,016 (732 - 1,900)	8.63x10 <sup>-11</sup>
Eotaxin	9	0	270	994 (488 - 1,573)	81 (28 - 126)	1.04x10 <sup>-10</sup>
MCSF	1	0	224	37,046 (18,575 - 74,373)	1,477 (1,194 - 2,670)	1.07x10 <sup>-10</sup>
CXCL6	5	0	226	1,579 (379 - 3,426)	18 (11 - 25)	1.22x10 <sup>-10</sup>
CXCL7	6	0	222	11,149 (5,773 - 20,656)	469 (87 - 646)	2.41x10 <sup>-10</sup>
CCL8	9	5	226	5,594 (1,569 - 13,625)	112 (89 - 207)	4.40x10 <sup>-10</sup>
CXCL9	4	1	226	3,896 (1,466 - 14,037)	122 (37 - 169)	5.62x10 <sup>-10</sup>
CCL7	12	0	270	1,138 (444 - 2,706)	114 (59 - 167)	7.53x10 <sup>-10</sup>
IL-12p40	14	0	270	571 (268 - 1,480)	69 (12 - 103)	1.00x10 <sup>-09</sup>
CCL3	14	1	269	6,113 (947 - 23,085)	125 (50 - 1,420)	1.09x10 <sup>-09</sup>
IL-2	7	0	270	60 (31 - 121)	4 (3 - 7)	1.63x10 <sup>-09</sup>
IL-18	9	0	267	12,206 (3,889 - 25,223)	706 (400 - 885)	2.25x10 <sup>-09</sup>
TGF-α	17	0	270	99 (47 - 176)	16 (10 - 26)	2.77x10 <sup>-09</sup>
sVCAM-1	2	0	270	238,145 (137,975 - 381,923)	42,310 (33,930 - 50,610)	3.66x10 <sup>-09</sup>
MIF	38	0	271	3,076 (1,242 - 9,653)	155 (139 - 170)	5.30x10 <sup>-09</sup>
IL-13	22	0	265	67 (38 - 134)	11 (5 - 28)	7.56x10 <sup>-09</sup>
CXCL11	8	2	226	507 (115 - 1,875)	14 (8 - 21)	1.25x10 <sup>-08</sup>
MMP-10	17	0	268	474 (256 - 890)	114 (65 - 190)	1.51x10 <sup>-08</sup>
VEGF	20	0	269	2,423 (1201 - 4,027)	499 (367 - 742)	1.92x10 <sup>-08</sup>

Supplementary Table 1: Continued

Analyte	<i>S. pneumoniae</i> meningitis				Negative controls	P-value
	%< LLOQ	%> ULOQ	N <sup>a</sup>	Median concentration	Median concentration	
Flt3Lig	21	0	270	382 (210 - 604)	71 (41 - 133)	3.36x10 <sup>-08</sup>
CX3CL1	23	0	269	1,720 (976 - 12,462)	534 (358 - 712)	4.61x10 <sup>-08</sup>
MMP-1	27	0	268	2,384 (871 - 5,891)	333 (158 - 594)	5.57x10 <sup>-08</sup>
TRAIL	31	0	226	57 (25 - 104)	10 (4 - 23)	1.83x10 <sup>-07</sup>
CCL5	16	0	270	148 (55 - 333)	20 (10 - 29)	1.87x10 <sup>-07</sup>
CCL15	25	0	226	372 (157 - 763)	88 (35 - 121)	2.25x10 <sup>-07</sup>
FGF2	27	0	270	1,429 (807 - 1,831)	484 (136 - 731)	3.48x10 <sup>-07</sup>
sCD40L	21	0	270	302 (160 - 598)	76 (50 - 104)	5.12x10 <sup>-07</sup>
sIL2Ra	41	0	270	179 (85 - 364)	55 (24 - 96)	2.05x10 <sup>-06</sup>
TNF-β	40	0	270	50 (24 - 515)	15 (10 - 24)	3.72x10 <sup>-06</sup>
CXCL13	45	0	226	12 (6 - 40)	3 (1 - 7)	3.74x10 <sup>-06</sup>
IL-7	43	0	269	255 (89 - 1,424)	92 (25 - 139)	2.18x10 <sup>-05</sup>
CCL14a	7	0	226	4,289 (2,232 - 7,500)	1,180 (938 - 1,803)	3.27x10 <sup>-05</sup>
CCL24	33	0	226	65 (22 - 142)	24 (10 - 29)	4.16x10 <sup>-05</sup>
IL-5	27	0	270	10 (6 - 15)	4 (2 - 7)	4.74x10 <sup>-05</sup>
IL-29	39	0	223	2,944 (1,765 - 4,002)	1,193 (474 - 2,122)	4.84x10 <sup>-05</sup>
PDGF-AB/BB	43	0	264	258 (104 - 570)	73 (48 - 123)	6.50x10 <sup>-05</sup>
IL-15	34	0	270	52 (26 - 83)	22 (15 - 34)	1.09x10 <sup>-04</sup>
CCL27	43	0	221	19 (10 - 41)	8 (6 - 12)	1.81x10 <sup>-04</sup>
CCL19	45	0	226	136 (78 - 328)	79 (38 - 98)	3.12x10 <sup>-04</sup>
CCL17	52	0	225	3 (2 - 5)	2 (1 - 3)	3.47x10 <sup>-03</sup>
EGF	65	0	270	139 (73 - 297)	81 (45 - 126)	4.27x10 <sup>-03</sup>
CCL22	49	0	270	1,031 (450 - 1,499)	707 (230 - 883)	4.59x10 <sup>-03</sup>
CCL26	97	0	226	89 (38 - 130)	41 (27 - 78)	0.01
CXCL5	58	0	226	854 (399 - 3,028)	577 (271 - 733)	0.01
LIF	100	0	224	426 (237 - 648)	636 (439 - 828)	0.01
IL-9	99	0	266	77 (41 - 122)	42 (22 - 83)	0.02
SCF	60	0	225	27 (14 - 52)	21 (10 - 27)	0.02
IL-21	88	0	224	34 (16 - 51)	20 (3 - 41)	0.02
CXCL12ab	89	0	224	2,771 (1,347 - 4,310)	1,992 (927 - 3,177)	0.11
XCL1	70	0	226	657 (343 - 1,342)	600 (202 - 750)	0.13
TSLP	99	0	224	63 (34 - 94)	45 (23 - 85)	0.24
IL-4	87	0	270	87 (45 - 122)	82 (25 - 116)	0.25
IL-11	82	0	223	2,524 (1,259 - 3,950)	2,129 (1,297 - 2,744)	0.25
IL-3	88	0	270	20 (10 - 34)	15 (7 - 29)	0.25
MMP-7	93	0	267	9,709 (4,511 - 15,483)	12,821 (7,112 - 16,053)	0.32
IL-16	82	0	224	353 (175 - 553)	280 (176 - 459)	0.38
IL-33	99	0	226	103 (48 - 161)	78 (34 - 148)	0.40
CCL13	100	0	226	284 (142 - 432)	333 (200 - 436)	0.41
IL-28A	95	0	225	71 (36 - 115)	68 (34 - 102)	0.50
IL-23	89	0	226	320 (135 - 462)	234 (143 - 443)	0.50
PDGF-AA	86	0	269	52 (26 - 73)	50 (21 - 73)	0.52
MMP-13	100	0	270	4,906 (2,822 - 7,730)	4,910 (1,618 - 8,411)	0.67
TPO	100	0	226	1,166 (629 - 1,694)	968 (328 - 1,747)	0.68
CCL1	100	0	226	45 (22 - 69)	47 (29 - 65)	0.71

Supplementary Table 1: Continued

Analyte	<i>S. pneumoniae</i> meningitis			Negative controls		P-value
	%< LLOQ	%> ULOQ	N <sup>a</sup>	Median concentration	Median concentration	
IL-20	98	0	225	1,529 (746 - 2,235)	1,489 (905 - 2,558)	0.74
MMP-12	99	0	270	8,076 (4,304 - 12,367)	7,784 (4,035 - 13,431)	0.90
MMP-2	99	0	268	23,466 (11,604 - 34,373)	26,267 (9,670 - 32,639)	0.93
CCL21	89	0	225	726 (269 - 1,069)	714 (482 - 922)	0.98

<sup>a</sup>Group size of pneumococcal meningitis patients varied between between 226 and 289 patients, depending on the analyte measured. The negative control group included 19 patients. Of pneumococcal meningitis patients the percentage of measurements below LLOQ of the assay and above the ULOQ are given, and the percentage of valid measurements. In the negative control group 100% of measurements were valid for all analytes. Data are given as median concentration in pg/ml with interquartile range and were compared with a MWU test of which the p-value is given in the last column. A Bonferroni-corrected P-value < 0.00057 was considered significant.

Supplementary table 2. Inflammatory mediators in the CSF of patients with favorable outcome compared to unfavorable outcome.

Analyte	Favorable outcome	Unfavorable outcome	p-value
MMP-1	1,530 (520 - 3,506)	4,441 (1,822 - 9,578)	4.95x10 <sup>-10</sup>
sICAM-1	50,530 (29,110 - 78,095)	78,580 (47,375 - 135,260)	9.36x10 <sup>-07</sup>
IL1RA	2,879 (905 - 6,777)	6,122 (3,325 - 11,492)	9.36x10 <sup>-06</sup>
CXCL5	681 (318 - 1,492)	2,410 (659 - 4,536)	1.41x10 <sup>-05</sup>
IL-10	1,204 (464 - 3,270)	2,787 (1,308 - 5,899)	2.15x10 <sup>-05</sup>
MMP-3	21,286 (6,487 - 77,267)	74,491 (16,062 - 159,301)	9.43x10 <sup>-05</sup>
CCL7	920 (377 - 2,042)	1,694 (726 - 3,955)	3.20x10 <sup>-04</sup>
MMP-10	432 (235 - 845)	619 (273 - 1,081)	1.16x10 <sup>-03</sup>
MIF	2,496 (1,138 - 6,768)	5,047 (1,522 - 17,345)	1.60x10 <sup>-03</sup>
CCL5	122 (48 - 273)	200 (85 - 417)	2.64x10 <sup>-03</sup>
CCL20	4,751 (1,774 - 12,819)	10,704 (2,863 - 22,370)	3.31x10 <sup>-03</sup>
TRAIL	46 (21 - 89)	74 (30 - 141)	3.88x10 <sup>-03</sup>
IL-6	70,748 (35,571 - 99,232)	91,726 (50,638 - 99,232)	0.01
CCL15	315 (138 - 604)	476 (233 - 964)	0.01
sVCAM-1	219,440 (121,682 - 335,125)	273,830 (159,437 - 492,420)	0.01
CXCL7	9,702 (4,863 - 18,389)	14,247 (7,726 - 22,684)	0.02
TGF- $\alpha$	91 (39 - 171)	120 (71 - 197)	0.02
CCL27	15 (9 - 33)	25 (12 - 51)	0.02
GCSF	14,937 (6,197 - 27,603)	20,372 (7,475 - 35,661)	0.02
IL-15	45 (24 - 78)	63 (36 - 103)	0.03
MCSF	34,290 (16,720 - 60,976)	53,020 (21,488 - 92,116)	0.03
IL-18	10,415 (3,077 - 22,697)	15,131 (6,474 - 28,401)	0.03
TPO	1,292 (672 - 1,727)	958 (611 - 1,491)	0.03
CCL4	13,173 (3,767 - 35,775)	20,628 (5,932 - 44,310)	0.04
FGF2	1,376 (749 - 1,726)	1,547 (904 - 1,984)	0.04
SCF	23 (13 - 42)	31 (15 - 67)	0.05
Flt3Lig	335 (196 - 546)	427 (245 - 696)	0.06
CXCL10	116,520 (52,534 - 251,953)	163,493 (83,790 - 360,925)	0.06
CCL8	4,700 (1,394 - 11,321)	7,652 (1,968 - 18,491)	0.08
CXCL12ab	2,690 (1,234 - 4,165)	3,220 (1,448 - 4,526)	0.08

Supplementary table 2. Continued

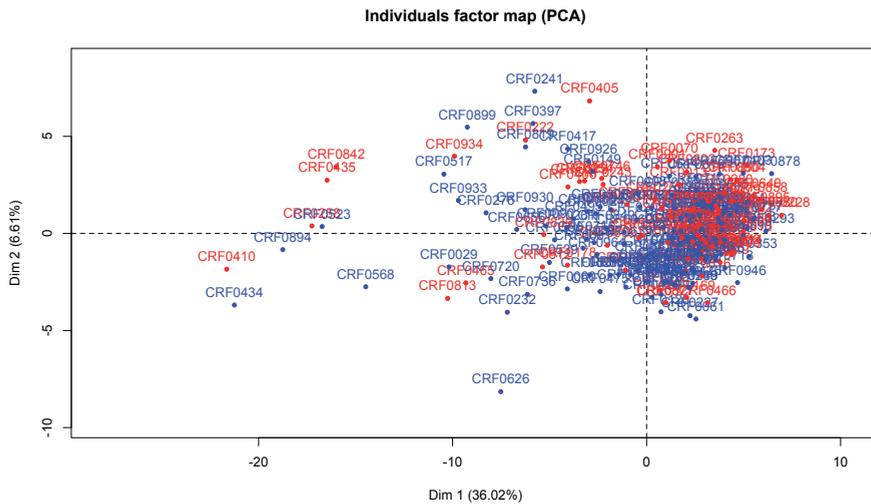
Analyte	Favorable outcome	Unfavorable outcome	p-value
CXCL6	1,373 (356 - 3,021)	1,935 (386 - 4,644)	0.08
TSLP	70 (37 - 96)	57 (31 - 84)	0.10
IL-12p40	530 (247 - 1,351)	627 (282 - 1,607)	0.12
CXCL9	3,728 (1,242 - 9,559)	4,206 (1,728 - 17,126)	0.13
IL-11	2,468 (1,221 - 3,682)	2,766 (1,370 - 4,430)	0.14
IL-3	22 (12 - 34)	18 (8 - 33)	0.15
CCL24	54 (20 - 143)	69 (40 - 128)	0.16
PDGF-AA	50 (23 - 70)	52 (29 - 77)	0.17
sIL-2Ra	179 (72 - 323)	179 (114 - 413)	0.18
VEGF	2,223 (1,130 - 3,806)	2,576 (1,244 - 4,639)	0.20
CCL21	713 (235 - 1,011)	746 (302 - 1,151)	0.22
CCL19	122 (77 - 297)	156 (78 - 388)	0.22
IL-12p70	127 (67 - 195)	150 (85 - 212)	0.22
IL-2	57 (26 - 118)	69 (31 - 131)	0.25
IFN- $\gamma$	1,212 (463 - 3,503)	1,401 (532 - 5,765)	0.25
MMP-12	8,461 (4,787 - 12,662)	7,690 (3,606 - 12,211)	0.27
TNF- $\alpha$	2,905 (409 - 11,945)	4,904 (726 - 11,351)	0.29
CXCL1	19,805 (4,033 - 39,171)	23,065 (5,836 - 42,783)	0.30
IL-21	32 (16 - 49)	37 (16 - 54)	0.31
IL-4	92 (51 - 123)	80 (41 - 118)	0.31
CXCL13	12 (6 - 31)	14 (6 - 44)	0.32
PDGF-AB/BB	258 (94 - 567)	263 (122 - 591)	0.32
IL-33	98 (44 - 144)	108 (56 - 172)	0.32
IL-17	143 (62 - 283)	169 (64 - 300)	0.37
CCL14a	4,295 (2,972 - 7,611)	4,284 (1,117 - 7,257)	0.38
CCL3	5,306 (887 - 21,963)	6,963 (1,146 - 25,251)	0.38
GMCSF	865 (323 - 1,583)	1,125 (261 - 1,951)	0.39
IFN $\alpha$ 2	1,151 (653 - 1,679)	1,214 (599 - 1,974)	0.39
CXCL11	434 (116 - 1,695)	595 (114 - 2,011)	0.42
MMP-7	9,663 (4,166 - 15,237)	9,911 (5,752 - 15,929)	0.43
IL-13	67 (31 - 135)	67 (38 - 134)	0.44
MMP-13	5,575 (2,759 - 7,910)	4,597 (2,892 - 6,988)	0.45
CCL26	89 (39 - 136)	88 (33 - 124)	0.47
CCL1	46 (22 - 70)	39 (21 - 69)	0.50
CCL2	99,802 (95,276 - 99,802)	99,802 (99,802 - 99,802)	0.50
IL-1 $\beta$	1,612 (258 - 6,022)	2,424 (388 - 5,807)	0.50
MMP-9	497,193 (497,193 - 497,193)	497,193 (497,193 - 497,193)	0.52
TNF- $\beta$	47 (23 - 501)	53 (25 - 547)	0.53
Eotaxin	956 (500 - 1,540)	1,065 (414 - 1,672)	0.55
CCL17	3 (2 - 5)	3 (1 - 6)	0.56
IL-23	311 (134 - 461)	338 (147 - 468)	0.58
IL-9	79 (42 - 127)	75 (39 - 114)	0.59
LIF	445 (191 - 667)	418 (272 - 638)	0.60
sCD40L	271 (158 - 580)	334 (163 - 652)	0.61
CCL13	305 (151 - 446)	275 (131 - 403)	0.69
CCL22	1,031 (475 - 1,499)	1,031 (387 - 1,499)	0.70
IL-29	2,882 (1,732 - 3,909)	3,076 (1,790 - 4,006)	0.73

Supplementary table 2. Continued

Analyte	Favorable outcome	Unfavorable outcome	p-value
IL-20	1,458 (684 - 2,235)	1,674 (796 - 2,354)	0.76
XCL1	673 (325 - 1,342)	630 (354 - 1,342)	0.76
IL-7	256 (89 - 1,388)	247 (81 - 1,516)	0.83
IL-5	10 (6 - 15)	10 (7 - 14)	0.86
EGF	137 (69 - 282)	139 (76 - 299)	0.88
MMP-2	22,812 (11,610 - 34,305)	24,634 (11,586 - 35,024)	0.88
IL-16	371 (175 - 554)	350 (170 - 551)	0.90
IL-28A	71 (36 - 115)	71 (32 - 116)	0.92
IL-1 $\alpha$	484 (223 - 1,275)	469 (165 - 1,415)	0.94
CX3CL1	1,755 (969 - 12,588)	1,678 (976 - 12,368)	0.99
CXCL8	53,241 (18,334 - 113,442)	54,127 (18,086 - 117,229)	1.00

Group size for patients with favorable outcome varied between 134 and 162 patients, and for unfavorable outcome between 87 and 109 patients, depending on the analyte measured. Data are given as median concentration in pg/ml with interquartile range and were compared with a MWU test of which the p-value is given in the last column. A Bonferroni-corrected P-value < 0.00057 was considered significant.

Supplementary figure 1. Principal component analysis.



Principal component analysis of patients with favorable outcome (blue) and unfavorable outcome (red) based on all 88 measured immune mediators. The principal component 1 (dim1) is given on the x-axis and principal component 2 (dim2) on the y-axis. CRF (case record form) are the patients individual study numbers.

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## SUMMARY AND GENERAL DISCUSSION

## Summary

Pneumococcal meningitis is a serious infectious disease of the meninges surrounding the brain and spinal cord, with high mortality and morbidity.<sup>1</sup> Despite treatment with antibiotics, adjuvant dexamethasone and advanced supportive care, 20% of patients do not survive pneumococcal meningitis.<sup>2</sup> There is a need for new adjuvant treatments to improve outcome. In this thesis we tried to obtain a better understanding of the pathophysiology of pneumococcal meningitis and to find new potential targets for future research aiming at new adjunctive therapies to improve outcome.

In Chapter 2 we reviewed the literature to date on the pathophysiology of pneumococcal meningitis. An adequate immune response is essential to effectively control infection and limit tissue damage as a result of bacterial growth and release of toxins.<sup>3-5</sup> Experimental studies showed that also the immune response itself can cause damage to the tissue of the host.<sup>6</sup> Dead pneumococci or purified pneumococcal constituents are able to induce a pronounced cerebrospinal fluid (CSF) inflammatory response, leukocyte influx, cerebral edema and increased intracranial pressure in experimental animals.<sup>6,7</sup> To limit host tissue damage as a result of inflammation, the host inflammatory response is controlled by an anti-inflammatory immune response that eventually down-regulates the inflammation. An effective immune response starts with the correct pattern recognition by the host innate immune system, thereby identifying the invading pathogen involved to initiate a tailored immune response.<sup>8,9</sup> Toll-like receptors (TLR's) have been extensively studied in pneumococcal meningitis and were found to play an important role, as TLR2 and 4 deficient mice showed low cytokine responses, higher bacterial loads and worse disease outcome.<sup>10-13</sup> A different group of pattern recognition receptors (PRR's) that was recently described include the Nod-like receptors (NLR's). NLR's are intracellular signaling molecules that have been reported to recognize several bacterial toxins, including pneumococcal pneumolysin and bacterial DNA.<sup>14-16</sup> In addition, two downstream effectors of inflammasome signaling, caspase-1 and IL-18 were shown to play a role in the brain damage associated with pneumococcal meningitis.<sup>17,18</sup> This fuelled our interest in these PRR's, which resulted in chapter 4 and 5.

In Chapter 3 we established an experimental mouse model of meningitis by intracisternal inoculation of *S. pneumoniae*. We aimed to develop an animal model that was reproducible, in which features of meningitis were on the foreground compared to systemic infection, and with as little as iatrogenic structural damage as possible. Bacterial meningitis was induced in all mice with good reproducibility and uniform disease course between mice. We measured cytokines and chemokines known to be involved in the immune response against pneumococci in humans and found CXCL1, CXCL2, IL-1 $\beta$  and IL-6 to be elevated in our mouse model. TNF- $\alpha$  and IL-10 were low in all body compartments measured. Furthermore, features of pneumonia and sepsis only became apparent at the end of the disease course. On histopathology of the brain we observed meningeal, parenchymal and perivascular lymphocytic infiltration, and

in some cases beginning abscess formation. Furthermore we found signs of microglial activation, hippocampal apoptosis and (micro) hemorrhages, which have also been described in meningitis patients.<sup>19-21</sup> However, we observed no signs of stroke, a well-known complication in human disease.<sup>22-25</sup>

In Chapter 4 we studied the role of the NACHT, LRR and PYD domains-containing protein (NLRP)-3 inflammasome in the pathophysiology of pneumococcal meningitis. We found the inflammasome downstream effector cytokines IL-1 $\beta$  and IL-18 in patients' CSF to be associated with systemic complications, and observed that cytokine levels were dependent on the causative *S. pneumoniae* serotype. Then, we induced pneumococcal meningitis in mice deficient for NLRP3, a protein involved in the NLRP3 inflammasome signaling pathway, and in mice lacking Apoptosis-associated Speck-like protein containing CARD (ASC), which is an adaptor protein of multiple inflammasome pathways. *Nlrp3*<sup>-/-</sup> mice showed less meningeal neutrophil influx and cerebral hemorrhages, which surprisingly was independent of ASC, IL-1 $\beta$  or IL-18. Both *Nlrp3*<sup>-/-</sup> and *Asc*<sup>-/-</sup> mice had a reduced systemic inflammatory response. The decrease of systemic inflammation was most pronounced in *Asc*<sup>-/-</sup> mice, and was accompanied by lower levels of brain pro-inflammatory cytokines CXCL1, CXCL2 and IL-6 at 30h after infection. We found no differences in IL-1 $\beta$  and IL-18 levels between wild-type (WT) and *Nlrp3*<sup>-/-</sup> or *Asc*<sup>-/-</sup> mice, which was unexpected. Our results implicate a role for inflammasome proteins NLRP3 and ASC in the regulation of the systemic inflammatory response and the development of cerebral damage during pneumococcal meningitis.

In Chapter 5 we analyzed eight nonsynonymous single nucleotide polymorphisms (SNP's) in coding regions of in genes involved in the inflammasome-signaling pathway, for which a commercial assay was available and with a reported minor allele frequency of at least 5%. A genetic variant in the *CARD8* gene was associated with unfavorable outcome in pneumococcal meningitis, which seemed to be driven by systemic and neurologic complications. The SNP in the *CARD8* gene was not associated with CSF IL-1 $\beta$  or IL-18 levels. The role of *CARD8* as a component of the NLRP3 inflammasome remains ambiguous, *CARD8* independent NLRP3 signaling has been described and recently *CARD8* was suggested to have an inhibitory effect on NLRP3 signalling.<sup>26,27</sup> Furthermore, *CARD8* plays a role in multiple other signaling pathways.<sup>28-31</sup> Thus, we found a correlation between a genetic variant in *CARD8* and unfavorable outcome, but further research is needed to elucidate the causality of the association and underlying mechanism.

In Chapter 6 we show that the lack of a *S. pneumoniae* *argGH* locus is associated with favorable outcome in patients with pneumococcal meningitis. The genes located on this locus are *argG* and *argH*, which are involved in the biosynthesis of arginine out of citrulline. Pneumococci are able to take up arginine from their surrounding via an ABC-transporter system that is present in all known pneumococcal strains. Only a minority of strains possess an arginine synthesis system.<sup>32</sup> Arginine is used for growth, but is also a precursor in the biosynthesis of polyamines, which have been implicated in oxidative stress responses and protection against free radicals.<sup>33</sup> We showed that a serotype 2

pneumococcal knock out strain for the locus (*D39ΔargGH*) is less resistant to H<sub>2</sub>O<sub>2</sub> *in vitro* compared to wild-type D39 and has impaired growth in human blood and CSF, but not in growth medium. Bacterial growth was attenuated in a meningitis and pneumonia model in mice inoculated with the *D39ΔargGH* pneumococcus, and in line with these results, *D39ΔargGH* inoculated mice had better survival. Thus, defective pneumococcal arginine synthesis results in decreased virulence and bacterial growth in blood and CSF in mice, which may be due to impaired growth as a result of arginine deficiency, decreased resistance to free radicals, or a combination of these.

In Chapter 7 we conducted an explorative study in which we analyzed 88 different inflammatory mediators in the CSF of patients with pneumococcal meningitis and related these to disease outcome. Seven inflammatory mediators were associated with unfavorable outcome, and included two matrix metalloproteinases (MMP-1 and MMP-3), an adhesion molecule (sICAM-1), two chemokines (CCL7 and CXCL5) and two anti-inflammatory cytokines (IL-1RA and IL-10). Of these MMP-1 and sICAM-1 were also associated with mortality and systemic complications. These data suggest a role for these inflammatory mediators in the pathophysiology of pneumococcal meningitis.

## Discussion

The main objective of this thesis was to gain more insight in the pathophysiology of pneumococcal meningitis. A more close understanding of the pathophysiology is needed to identify potential new targets for future studies aiming at the development of adjunctive therapies that can reduce brain damage and improve outcome of pneumococcal meningitis.

In chapter 3 we describe how we set up an experimental mouse model of pneumococcal meningitis. We choose for intracisternal inoculation as route of infection. The advantages of this model are its reproducibility, as infection proceeds to meningitis in all mice with similar CSF bacterial loads and disease progression over time. A drawback of this model is that the natural route of infection, via the lungs and blood, is bypassed and the systemic immune response is activated after induction of meningitis rather than before. Other studies using intraperitoneal or intranasal inoculation resemble the natural route of infection more closely, however not all mice develop meningitis and a proportion of animals succumb from sepsis and pneumonia.<sup>34-36</sup> In our study, signs of respiratory distress and positive blood cultures only became apparent at the end of the disease course, which makes the model suitable for studying meningitis. On histopathology of the brain we observed multiple features resembling human disease, except for signs of stroke, a well-known complication in human disease and described in 17% of patients with pneumococcal meningitis.<sup>22-25</sup> Cortical necrosis has been described in experimental models using rats or infant mice, but not in adult mice.<sup>37,38</sup> Thus, our model resembles the human situation in many ways, but also shows several differences, which need to be considered when interpreting the results of future experiments using this mouse model.

In Chapter 4 we evaluated the role of the inflammasome pathway in a mouse model of pneumococcal meningitis. First, we showed that in experimental meningitis NLRP3 signaling was responsible for an enhanced neutrophil influx and cerebral hemorrhages, which was independent of ASC, IL-1 $\beta$  or IL-18. Inflammasome independent NLRP3 signaling has been described before in different disease models, but its precise mechanism is not yet clear.<sup>39,40</sup> Secondly, in our study both NLRP3 and ASC deficiency resulted in decreased systemic bacterial loads and inflammatory response, which was most pronounced in *Asc*<sup>-/-</sup> mice, that also showed decreased brain cytokine and chemokine levels. Surprisingly, we found no differences in IL-1 $\beta$  and IL-18 levels between knock out and wild-type mice. Possible explanations for this unexpected finding can be that we measured at an inadequate time point, as IL-1 $\beta$  in humans is increased in the first 18 hours of infection<sup>41</sup>, or that the serotype 3 pneumococcus is a relatively weak activator of inflammasome signaling compared to other serotypes. Our results are conflicting with a study that was concurrently done, in which meningitis was induced in *Asc*<sup>-/-</sup> and *Nlrp3*<sup>-/-</sup> mice with a serotype 2 pneumococcus.<sup>42</sup> *Asc*<sup>-/-</sup> and *Nlrp3*<sup>-/-</sup> mice had a better clinical outcome and decreased brain inflammation, which was dependent on IL1- $\beta$  and IL-18 signaling.<sup>42</sup> These different outcomes may be the result of the different pneumococcal serotypes used in the experiments. Pneumococcal serotype 3 is more heavily encapsulated and more virulent than serotype 2. In line with this hypothesis we show that CSF IL-1 $\beta$  production in patients differs depending on the pneumococcal serotype, and is relatively low with serotype 3. We were unable to show this for serotype 2 as this is not a causative agent of pneumococcal meningitis in humans.<sup>1</sup> However, in *in vitro* experiments high doses of serotype 2 pneumococci were needed to induce IL- $\beta$  production.<sup>43</sup> The inflammasomes are relatively newly discovered pathogen recognition receptors and part of a complicated system of which the knowledge is rapidly expanding. In conclusion, these results implicate a role for inflammasome signaling in the pathogenesis of pneumococcal meningitis, however the mechanism appears to be different than previously described for the NLRP3 signaling pathway, which warrants further research.

In Chapter 5 we analyzed eight SNP's in genes involved in multiple inflammasome pathways, and correlated these to disease outcome. We found a genetic variant in the *CARD8* gene to be associated with unfavorable outcome, also in the subgroup of patients with pneumococcal meningitis. Secondly, we found that a SNP in NLRP1 was associated with mortality in pneumococcal meningitis, although the association was not preserved after correction for multiple testing. Therefore, confirmation of this association in a larger cohort or an experimental study is needed. *CARD8* was initially described as a negative regulator of NF- $\kappa$ B, caspase-1 dependent IL-1 $\beta$  production, Nod2 activation and apoptosis.<sup>28-31</sup> The role of *CARD8* in the NLRP3 inflammasome complex remains ambiguous.<sup>44</sup> Some studies described *CARD8* as a component of the NLRP3 inflammasome, however *CARD8* independent NLRP3 inflammasome activation has also been described and more recent studies showed a possible role for *CARD8* as a negative regulator of the NLRP3 inflammasome signaling.<sup>28,45,26</sup> The genetic variant described is known to result

in deficient protein synthesis of CARD8, and as CARD8 is a negative regulator of several different inflammatory pathways including NLRP3 inflammasome signaling, presence of the genetic variant would therefore be predicted to have pro-inflammatory effects.<sup>27,28</sup> Although we found an association with disease outcome and systemic complications we did not find any associations of the *CARD8* SNP with CSF IL-1 $\beta$  or IL-18 levels. Since CARD8 has several other functions we cannot draw conclusions regarding the causality or mechanism from this study, and further research is needed to answer these questions.

In Chapter 6 we show that the lack of a *S. pneumoniae* arginine biosynthesis system (*argGH* locus) is associated with decreased bacterial growth and susceptibility to oxidative stress, and favorable disease outcome. *S. pneumoniae* is mainly auxotrophic for arginine, only a minority of strains (<40%) possess an arginine synthesis system. The only arginine synthesis system described to date in the pneumococcus is encoded by the *argG* and *argH* genes.<sup>32</sup> Since the *argGH* locus is present in a minority of pneumococcal isolates, it could represent a virulence factor that has unintentionally led to increased virulence leading to invasive disease and death of the host thereby limiting the chances on transmission to other hosts and further expansion of the phenotype. This ‘avirulence hypothesis’ has been posed also for other pathogens, and assumes that pathogens pursue avirulence, but in the co-evolution with their niche, acquire new virulence factors that coincidentally lead to invasive disease of the host thereby impairing its chances on survival and transmission to new hosts and extinction of the phenotype.<sup>46</sup>

In Chapter 7 we found that seven inflammatory mediators were associated with unfavorable outcome in patients with pneumococcal meningitis. These included sICAM-1, MMP-1, MMP-3, CCL7, CXCL5, IL-1RA and IL-10. The first two were also associated with mortality. Levels of inflammatory mediators correlated with parameters of severity of infection such as high CSF protein count and low CSF-blood glucose ratio. Surprisingly we found no association of any of the inflammatory mediators with CSF leukocyte count, which suggests that local cells types play a role in the production of these proteins, or that they were produced in the systemic compartment and crossed a leak blood brain barrier. Soluble ICAM-1 is suggested to either be released as a result of tissue damage, promoting infection, or to play a role in inhibiting the LFA-1 receptor and related signaling, thereby decreasing leukocyte influx to the site of infection.<sup>47</sup> We found no correlation between sICAM-1 levels in CSF and CSF leukocyte count, which suggests its mechanism in pneumococcal meningitis is different from leukocyte influx. Furthermore, we identified two MMP's to be involved with outcome. In the last years the role of MMP's as immune-modulating proteases next to their known function as modulators of the extracellular matrix, has become more evident.<sup>48</sup> Not much is known about the role of MMP-1 or MMP-3 in pneumococcal infection. Substrates of MMP-1 include multiple inflammatory components such as pro-TNF, IL-1 $\beta$ , and several chemokines of which some are cleaved into receptor antagonists and may exert an anti-inflammatory effect.<sup>49</sup> IL-10 and IL-1RA are anti-inflammatory cytokines, important in impairing tissue damage in infection.<sup>50</sup> IL-10 levels were highly correlated to IL-1RA. IL-1RA is an antagonist of the IL-1 receptor, preventing the signaling

of both IL-1 $\alpha$  and IL-1 $\beta$ . In our study, IL-1RA/IL-1 $\beta$  ratios were well below the levels needed to antagonize IL-1 $\beta$ . Either this reflects an insufficient attempt to antagonize IL-1R signaling or possibly IL-1RA exerts different functions besides inhibiting IL-1R signaling. Recently, IL-1RA was described to inhibit NLRP3 inflammasome signaling.<sup>51</sup> To find high levels of two anti-inflammatory proteins to be associated with unfavorable outcome was unexpected. Possibly this reflects the failing control of the inflammatory response or that an overactive anti-inflammatory response may lead to ineffective infection control and bacterial clearance, and unfavorable outcome. CCL7 and CXCL5 are both chemokines that attract neutrophils, promoting inflammation. In a pneumococcal pneumonia model CCL7 was shown to exhibit a pro-inflammatory effect.<sup>52</sup> However, CCL7 can be modulated in a receptor antagonist resulting in an inverse inflammatory effect. The assay used to measure CCL7 does not discriminate between the agonistic and antagonistic variants. In previous reports CXCL5 was demonstrated in the CSF of patients with bacterial meningitis where it exerts a chemotactic activity towards neutrophils.<sup>53</sup>

The limitations of this study include an inclusion bias (only patients with positive CSF culture, severely ill patients may have a contra-indication for lumbar puncture) and differences in sample handling. We cannot draw conclusions whether these inflammatory mediators are produced intrathecally or pass the blood brain barrier from the systemic compartment, and how these levels are correlated to the CSF bacterial load. For future experiments analysis of inflammatory mediators in paired blood samples and measurement of CSF bacterial loads would be interesting. Furthermore, we cannot draw conclusions from this study about a causative relationship between the inflammatory mediators found and unfavorable outcome. Therefore, further studies on the role of these inflammatory mediators in pneumococcal meningitis are needed.

### Future research

Further research will focus on the hypothesis free identification of pathways and biological processes involved in disease outcome of pneumococcal meningitis. For this purpose patient clinical data, genetic data, bacterial genetic data and CSF inflammatory mediators will be analyzed altogether. As part of the MeninGene study we collected DNA samples of a large proportion of patients, and in 198 cases we have paired CSF samples that were available for analysis of immune mediators and this number is still expanding. We have sequenced a large set of exome variants in these DNA samples and will correlate this data with the levels of CSF inflammatory mediators. In all cases the causative agent was isolated and stored. Genetic analysis of the causative *S. pneumoniae* strain combined with patient data may lead to a better understanding of host-pathogen interactions. This hypothesis free approach will hopefully lead to the identification of more proteins or pathways involved in the pathophysiology of pneumococcal meningitis.

Secondly, it would be interesting to broaden our research on the inflammatory mediators detected in CSF of patients. Whether they are also expressed in the mouse, in what cell types and to assess whether these inflammatory mediators are involved in, or

are just markers of, unfavorable outcome of disease. The last question could be addressed using a comparative study with knock out mice in our pneumococcal mouse model. Then, it would be interesting to go into more detail and assess whether these inflammatory mediators are in their agonistic or antagonistic state, where applicable, and gain more insight in their function and role in the inflammatory response. Since we found two anti-inflammatory cytokines (IL-10 and IL-1RA) to be associated with unfavorable outcome in patients, it would be interesting to take a specific look at the role of regulatory T-cells, which are known for their ability to suppress the inflammatory response. In past years it became more evident that in the elderly, which have a high incidence of pneumococcal meningitis, regulatory T-cell function and therefore immunosuppression increases.<sup>54,55</sup> Furthermore, to gain more insight in the level of bacterial control in patients in relation to the anti-inflammatory mediators that were found in the CSF, and whether the CSF inflammatory mediators are produced in the CSF or enter the CNS from the periphery, it would be relevant to collect data on the CSF bacterial load and peripheral blood levels of inflammatory mediators in our patient cohort.

In conclusion, we found that inflammasome signaling was associated with systemic complications, and that inflammasome independent NLRP3 signaling induced brain damage in our mouse model of pneumococcal meningitis. Furthermore we showed that pneumococcal virulence factors play a role in the clinical outcome of patients with pneumococcal meningitis, as the absence of an arginine synthesis pathway in the causative *S. pneumoniae* strain is associated with favorable outcome in these patients. Lastly, we found two MMP's, two anti-inflammatory cytokines, two chemokines and a signaling molecule in the CSF to be associated with disease outcome in patients, suggesting a role for these inflammatory mediators in the pathophysiology of pneumococcal meningitis. Further research will be focusing on combining patient clinical and genetic data with genetic data of the causative *S. pneumoniae* strains and the levels of CSF inflammatory mediators, to gain more insight in the host-pathogen interaction, and its effects on the inflammatory response and patient outcome. We hope this will lead to the identification of new pathways or biological processes involved in the outcome of pneumococcal meningitis, which can be further explored in the pneumococcal meningitis mouse model to find potential new targets for adjunctive therapies, and will eventually get us back from bench to bedside.

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## NEDERLANDSE SAMENVATTING

## Samenvatting

Pneumokokken meningitis is een ernstige infectieziekte waarbij de hersenvliezen die de hersenen en het myelum omgeven geïnfecteerd zijn. De ziekte heeft een hoge mortaliteit en morbiditeit<sup>1</sup>. Twintig procent van de patiënten overlijdt, ondanks behandeling met antibiotica in combinatie met dexamethason, en de huidige geavanceerde intensive-care zorg.<sup>2</sup> Er is dringend behoefte aan nieuwe adjuvante therapieën om de uitkomst van patiënten met pneumokokken meningitis te verbeteren. In dit proefschrift trachten we een beter inzicht te krijgen in de pathofysiologie van pneumokokken meningitis, met als uiteindelijk doel om nieuwe potentiële behandelstrategieën te identificeren voor de ontwikkeling van een nieuwe adjuvante therapie om de ziekte uitkomst te verbeteren.

Hoofdstuk 2 van dit proefschrift geeft een overzicht van de huidige literatuur over de pathofysiologie van pneumokokken meningitis. Om schade aan het zenuwstelsel te voorkomen als gevolg van de bacteriële infectie en de toxines die hierbij vrijkomen, is een effectieve immuunrespons essentieel.<sup>3-5</sup> In eerdere studies is aangetoond dat naast de bacterie ook de immuunrespons van de gastheer weefselschade kan veroorzaken<sup>6</sup>. Dode pneumokokken of gezuiverde eiwitten of polysaccharides van de pneumokok, kunnen een forse immuunrespons teweeg brengen in de liquor bij proefdieren, die leidt tot leukocyten influx, hersenoedeem en verhoogde intracranieële druk.<sup>6,7</sup> Om dit te voorkomen wordt de immuunrespons van nature in bedwang gehouden door een anti-inflammatoire reactie die er uiteindelijk voor zorgt dat de immuunrespons uitdooft. Een effectieve immuunrespons begint bij de juiste patroon herkenning van het infecterende pathogeen door het innate immuun systeem, waardoor de juiste afweerreactie gericht op dat specifieke pathogeen op gang komt.<sup>8,9</sup> Toll-like receptoren (TLR's) zijn pattern recognition receptoren (PRR's) die een belangrijke rol spelen in pneumokokken meningitis. TLR2 en 4 deficiënte muizen hebben een lagere cytokine response, hogere bacteriële load en slechtere ziekte uitkomst.<sup>10-13</sup> Een andere groep PRR's zijn de Nod-like receptoren (NLR's) die relatief recent in de literatuur zijn beschreven. Dit zijn intracellulaire signalerings eiwitten die onder andere verschillende bacteriële toxines kunnen herkennen waaronder pneumokokken pneumolysine, maar ook bacterieel DNA.<sup>14-16</sup> Er is aangetoond dat twee down-stream effector moleculen van inflammasome activatie, caspase-1 en IL-18 een rol spelen in de hersenschade die ontstaat na pneumokokken meningitis.<sup>17-18</sup> Hierdoor werd onze interesse gewekt in deze groep PRR's, wat heeft geresulteerd in hoofdstuk 4 en 5.

In hoofdstuk 3 laten we zien hoe we een experimenteel meningitis muismodel hebben opgezet, waarbij pneumokokken intracisternaal worden geïnoculeerd. Ons doel was een diermodel te ontwikkelen dat reproduceerbaar is, en waarbij de meningitis component van de infectie op de voorgrond staat, in tegenstelling tot de systemische infectie, en dit door zo min mogelijk iatrogene schade te veroorzaken. Intracisternale injectie van pneumokokken leidde tot bacteriële meningitis in alle muizen, met een goede reproduceerbaarheid een uniform ziektebeloop tussen de muizen per groep. In ons model hebben we cytokines en chemokines gemeten waarvan bekend is dat ze een rol spelen in de immuun reactie tegen pneumokokken,

en we vonden verhoogde concentraties CXCL1, CXCL2, IL1 $\beta$  en IL-6. Echter, TNF- $\alpha$  en IL-10 waren niet verhoogd in de verschillende organen die we hebben bekeken. Tekenen van pneumonie en sepsis kwamen pas later in het ziekte beloop. Histopathologie (PA) van de hersenen toonde meningeale, parenchymateuze en perivasculaire lymfocyten infiltratie, en in enkele gevallen beginnende abces vorming. Verder waren er aanwijzingen voor microglia activatie, apoptose in de hippocampus en (micro) bloedingen, alle drie kenmerken die ook in patiënten met pneumokokken meningitis zijn beschreven.<sup>19-21</sup> Echter, we vonden geen herseninfarcten bij PA in ons muismodel, wat een belangrijke complicatie is in patiënten.<sup>22-25</sup>

In hoofdstuk 4 hebben we gekeken naar het NACHT, LRR and PYD domains-containing protein (NLRP)-3 inflammasome, en de rol hiervan in pneumokokken meningitis. In patiënten met meningitis bleken concentraties van IL-1 $\beta$  en IL-18 in de liquor geassocieerd te zijn met systemische complicaties, en deze concentraties waren gerelateerd aan het serotype pneumokok dat uit de liquor werd geïsoleerd. Vervolgens hebben we gekeken naar muizen met een deficiëntie voor NLRP3, een eiwit dat betrokken is in de NLRP3 signaling pathway, en in muizen met een deficiëntie voor Apoptosis-associated Speck-like protein containing CARD (ASC), wat een onderdeel is van meerdere inflammasome signaling pathways. *Nlrp3*<sup>-/-</sup> muizen hadden minder meningeale neutrofielen influx en hersenbloedingen op PA, wat onafhankelijk leek te zijn van ASC, IL-1 $\beta$  en IL-18. Zowel *Nlrp3*<sup>-/-</sup> en *Asc*<sup>-/-</sup> muizen produceerden lagere concentraties systemische inflammatoire cytokines. Dit was het meest uitgesproken in de *Asc*<sup>-/-</sup> muizen, en ging samen met een lagere concentratie CXCL1, CXCL2 en IL-6 in de hersenen op 30 uur na infectie. Tegen alle verwachting in werden geen verschillen gevonden in IL-1 $\beta$  en IL-18 concentraties tussen wild-type (WT) en *Nlrp3*<sup>-/-</sup> of *Asc*<sup>-/-</sup> muizen. Deze resultaten suggereren een rol voor NLRP3 en ASC in de systemische immunerespons en het ontstaan van hersenschade bij pneumokokken meningitis.

In hoofdstuk 5 hebben we acht nonsynonymous single nucleotide polymorfismen (SNP's) geanalyseerd in de coderende regionen van genen die geassocieerd zijn met de inflammasome signalerings cascade, en voor welke een commerciële assay beschikbaar was en met een gerapporteerde minor allel frequentie van minimaal 5%. Een genetische variant in het *CARD8* gen bleek geassocieerd te zijn met slechte uitkomst in patiënten met pneumokokken meningitis, wat leek gedreven te worden door zowel systemische als neurologische complicaties. Deze SNP was niet geassocieerd met concentraties IL-1 $\beta$  en IL-18 in de liquor. De rol van *CARD8* als onderdeel van de NLRP3 signalerings cascade is onduidelijk, en ook *CARD8* onafhankelijke NLRP3 signalering en recent zelfs inhibitie van NLRP3 signalering door *CARD8* zijn in de literatuur beschreven.<sup>26,27</sup> Daarbij speelt *CARD8* ook een rol in verschillende andere signalerings cascades.<sup>28-31</sup> Concluderend vonden we een correlatie tussen een genetische variant in het *CARD8* gen met slechte ziekte uitkomst, verder onderzoek is nodig om de causaliteit van deze relatie en het onderliggend mechanisme aan te tonen.

In hoofdstuk 6 laten we zien dat *S. pneumoniae* isolaten die het *argGH* locus missen zijn geassocieerd met een relatief goede ziekte uitkomst bij de patiënt. De genen op het *argG*

en *argH* locus zijn betrokken bij de biosynthese van citrulline in arginine. Pneumokokken kunnen arginine opnemen uit hun omgeving via een ABC-transporter systeem dat in alle pneumokokken stammen die beschreven zijn aanwezig is. Slechts een minderheid van de pneumokokken stammen hebben een systeem waarmee arginine kan worden geproduceerd.<sup>32</sup> Arginine wordt door de pneumokok gebruikt voor groei, maar is ook een voorloper van de biosynthese van polyamines, die een rol spelen in de oxidatieve stress reactie en de bescherming tegen vrije radicalen.<sup>33</sup> We toonden aan dat een serotype 2 knock out pneumokok voor het *argGH* locus (*D39ΔargGH*) gevoeliger is voor H<sub>2</sub>O<sub>2</sub> *in vitro* vergeleken met een WT D39 en dat deze knock out variant een slechtere overleving had in bloed en liquor, maar niet in kweekmedium. Verder groeide de knock out variant slechter in een muizen meningitis en pneumonie model, en de muizen geïnoculeerd met de knock variant hadden een betere overleving. Concluderend, leidt een defect in het pneumokokken arginine synthetase systeem tot verminderde virulentie en bacteriegroei in bloed en liquor in muizen, wat mogelijk wordt veroorzaakt door een slechtere groei als gevolg van arginine deficiëntie, verhoogde gevoeligheid voor oxidatieve stress of een combinatie van beiden.

In hoofdstuk 7 hebben we in een exploratieve studie 88 inflammatoire mediators geanalyseerd in de liquor van patiënten met pneumokokken meningitis en gerelateerd aan ziekte uitkomst. Zeven inflammatoire mediators waren geassocieerd met slechte ziekte uitkomst, waaronder twee matrix metalloproteinases (MMP-1 en MMP-3), een adhesie molecuul (sICAM-1), twee chemokines (CCL7 en CXCL5) en twee anti-inflammatoire cytokines (IL-1RA en IL-10). Hiervan waren MMP-1 en sICAM-1 ook geassocieerd met mortaliteit en systemische complicaties. Deze resultaten suggereren een rol in de pathofysiologie van pneumokokken meningitis voor deze inflammatoire mediators.

## Discussie

Het doel van dit proefschrift was een beter inzicht te krijgen in de pathofysiologie van pneumokokken meningitis. Een beter begrip van de pathofysiologie is belangrijk om zo nieuwe potentiële behandelstrategieën voor de toekomst te identificeren, met als uiteindelijk doel om nieuwe adjuvante behandelingen te ontwikkelen en zo de uitkomst van patiënten met pneumokokken meningitis te verbeteren.

In hoofdstuk 3 beschrijven we het opzetten van een experimenteel muismodel voor pneumokokken meningitis. We kozen voor intracisternale inoculatie als techniek om meningitis te induceren. De voordelen van dit model zijn de reproduceerbaarheid, de infectie veroorzaakt meningitis in alle muizen met een gelijke bacterie load in de liquor en ziekte progressie in de tijd. Een nadeel van dit model is dat de natuurlijke infectieroute, via de longen en bloed worden gepasseerd, en dat de systemische immuun respons daardoor pas op gang komt na het ontstaan van de meningitis in plaats van ervoor. In andere studies waarin intraperitoneale of intranasale inoculatie wordt gebruikt hebben meer

overeenkomsten met de natuurlijke route van infectie, echter hebben als nadeel dat niet alle muizen meningitis ontwikkelen en een deel bezwijkt aan een pneumokokken pneumonie of sepsis.<sup>34-36</sup> In onze studie komen respiratoire insufficiëntie en positieve bloedkweken pas later in het ziektebeloop, wat dit model geschikt maakt voor het bestuderen van meningitis. PA van de hersenen liet meerdere kenmerken zien die ook bij patiënten worden gevonden, behalve herseninfarcten wat een belangrijke complicatie is in patiënten en in 17% van de patiënten met pneumokokken meningitis wordt beschreven.<sup>22-25</sup> Corticale necrose is wel eerder beschreven in meningitis modellen in ratten of zeer jonge muizen, maar niet in volwassen muizen.<sup>37,38</sup> Concluderend, zijn er verschillende kenmerken van ons muismodel die overeenkomen met de kenmerken die worden beschreven in patiënten, maar zijn er ook enkele verschillen die in acht moeten worden genomen wanneer men de resultaten interpreteert die verkregen zijn met dit muismodel.

In hoofdstuk 4 evalueren de rol van de inflammasome signaleringscascade in een pneumokokken meningitis muis model. Eerst tonen we aan dat in experimentele meningitis NLRP3 signalering verantwoordelijk is voor een verhoogde neutrofielen influx en cerebrale bloedingen, wat onafhankelijk bleek te zijn van ASC, IL-1 $\beta$  of IL-18. Inflammasome onafhankelijke NLRP3 signalering is eerder in de literatuur beschreven, in verschillende ziekte modellen, maar het onderliggende mechanisme is nog niet geheel opgehelderd.<sup>39,40</sup> Ten tweede, toonden we aan dat zowel NLRP3 als ASC deficiëntie leiden tot minder systemische bacteriële uitgroei en inflammatie wat het meest uitgesproken was in de *Asc*<sup>-/-</sup> muizen, waarin ook de cerebrale cytokine levels lager waren. Verassend genoeg vonden we geen verschillen in IL-1 $\beta$  en IL-18 concentraties in knock out en WT muizen. Een mogelijke verklaring voor dit onverwachte resultaat is dat we niet op het juiste tijdstip hebben gemeten, gezien bekend is uit de literatuur dat IL-1 $\beta$  in patiënten in de eerste 18 uur van infectie verhoogd is.<sup>41</sup> Of dat serotype 3 pneumokokken relatief zwakke activators zijn van de inflammasome signalerings cascade in vergelijking met andere serotypen. De resultaten zijn tegenstrijdig met de resultaten van een studie die gelijktijdig is gedaan, en waarin meningitis werd geïnduceerd in *Asc*<sup>-/-</sup> en *Nlrp3*<sup>-/-</sup> muizen met een serotype 2 pneumokok.<sup>42</sup> *Asc*<sup>-/-</sup> en *Nlrp3*<sup>-/-</sup> muizen hadden een betere klinische uitkomst en minder tekenen van inflammatie in de hersenen, welke afhankelijk waren van IL-1 $\beta$  en IL-18.<sup>42</sup> De verschillen tussen de studies zouden kunnen worden verklaard door de verschillende pneumokokken serotypen die zijn gebruikt. Serotype 3 heeft een dikker kapsel en is virulenter dan de serotype 2 pneumokok. We toonden aan dat IL-1 $\beta$  concentraties in de liquor van patiënten verschilt met het serotype pneumokok dat de meningitis veroorzaakt, en relatief laag is bij serotype 3 pneumokokken, wat deze hypothese ondersteunt. Voor serotype 2 kon dit niet worden aangetoond omdat dit geen verwekker is van pneumokokken meningitis in mensen.<sup>1</sup> Echter, in *in vitro* experimenten waren hoge doseringen serotype 2 pneumokokken nodig om IL-1 $\beta$  productie te induceren.<sup>43</sup> De inflammasomes zijn relatief nieuw ontdekte PRR's, en onderdeel van een gecompliceerd signalerings systeem waar snel veel nieuwe literatuur over beschikbaar komt. Concluderend, impliceren deze resultaten een rol voor de

inflammasome signalerings cascade in de pathogenese van pneumokokken meningitis, echter het mechanisme lijkt anders te zijn dat de meest gangbare beschreven NLRP3 signalerings cascade, en vervolg onderzoek is nodig om dit verder uit te diepen.

In hoofdstuk 5 hebben we acht SNP's bepaald in genen die betrokken zijn bij inflammasome signalering, en we hebben deze gecorreleerd aan de ziekte uitkomst in patiënten. We vonden een genetische variant in het *CARD8* gen welke was geassocieerd met slechte ziekte uitkomst, ook in de subgroep van patiënten met pneumokokken meningitis. Ten tweede vonden we een SNP in het *NLRP1* gen welke was geassocieerd met mortaliteit in pneumokokken meningitis, echter de associatie bleef niet significant na correctie voor multiële testen. Bevestiging van een associatie is nodig in een grotere groep patiënten of een experimentele studie. *CARD8* is initieel beschreven als een negatieve regulator van NF- $\kappa$ B, caspase-1 afhankelijke IL-1 $\beta$  productie, Nod2 activatie en apoptose.<sup>28-31</sup> De rol die *CARD8* speelt in het inflammasome complex is nog niet helemaal opgehelderd.<sup>44</sup> Er zijn studies die *CARD8* beschrijven als onderdeel van het NLRP3 inflammasome, maar ook *CARD8* onafhankelijke NLRP3 signalering is beschreven, en een recente studie toonde *CARD8* als een mogelijke negatieve regulator van het NLRP3 inflammasome.<sup>28,45,26</sup> De genetische variant die in onze studie is beschreven, resulteert in een eiwit deficiëntie, en als *CARD8* een negatieve regulator is van meerdere inflammatie cascades, inclusief het NLRP3 inflammasome, zou deze genetische variant een pro-inflammatoir effect moeten hebben.<sup>27,28</sup> We vonden een associatie met ziekte uitkomst en systemische complicaties, echter we vonden geen associatie tussen de *CARD8* SNP en IL-1 $\beta$  en IL-18 concentraties in de liquor. Aangezien *CARD8* ook meerdere andere functies heeft, kunnen we geen conclusie trekken uit deze studie over de causaliteit van de associatie of het mechanisme, daarvoor is verder onderzoek nodig.

In hoofdstuk 6 laten we zien dat afwezigheid van een *S. pneumoniae* arginine biosynthese systeem (het *argGH* locus) geassocieerd is met een verlaagde bacteriële uitgroei, verhoogde gevoeligheid voor oxidatieve stress en een relatief goede ziekte uitkomst van de patiënt. *S. pneumoniae* is auxotroof wat betreft arginine, en slechts een minderheid van het aantal pneumokokken stammen (<40%) heeft een arginine synthetase systeem. De *argG* en *argH* genen zijn tot op heden het enige arginine synthetase systeem beschreven in pneumokokken.<sup>32</sup> Omdat het *argGH* locus aanwezig is in de minderheid van de pneumokokken isolaten, zou het een virulentie factor kunnen betreffen die onbedoeld heeft geleid tot een verhoogde neiging tot invasieve ziekte en mortaliteit van de host. Hierdoor benadeelt de pneumokok zichzelf omdat de kans op transmissie naar een volgende gastheer en verdere expansie van het fenotype wordt verhinderd. Deze 'a-virulentie hypothese' is van toepassing op meerdere pathogenen en gaat ervanuit dat deze pathogenen a-virulentie nastreven, maar in de co-evolutie met hun niche nieuwe virulentiefactoren bemachtigen die per toeval leiden tot invasieve ziekte van de gastheer en daarmee de kans op overleving en transmissie van het pathogeen kleiner maken, wat uiteindelijk leidt tot uitsterven van het fenotype.<sup>46</sup>

In hoofdstuk 7 tonen we zeven inflammatoire mediators aan in de liquor die geassocieerd zijn met slechte ziekte uitkomst bij patiënten met pneumokokken

meningitis. Dit zijn sICAM-1, MMP-1, MMP-3, CCL7, CXCL5, IL-1RA en IL-10. De eerst twee genoemde waren ook geassocieerd met mortaliteit. Concentraties van deze inflammatoire mediators correleerden met parameters voor ernst van de infectie zoals een hoog liquor eiwit en lage liquor-bloed glucose ratio. Tot onze verbazing vonden we geen associatie tussen de inflammatoire mediators en liquor leukocyten getal, wat de suggestie wekt dat lokale cellen in de subarachnoïdale ruimte en het ventrikelsysteem een rol spelen in de productie van deze inflammatoire mediators, of dat ze in het systemische compartiment zijn geproduceerd en door een lekke bloed-hersenbarrière naar de liquor zijn gediffundeerd. Over soluble ICAM-1 wordt gesuggereerd dat het mogelijk vrijkomt bij weefselschade als gevolg van infectie of dat het een rol speelt bij de inhibitie van de LFA-1 receptor, en er voor zorgt dat er minder leukocyten influx is naar de plaats van infectie.<sup>47</sup> Wij vonden geen correlatie tussen sICAM-1 concentratie en het leukocyten getal in de liquor, wat suggereert dat de functie van sICAM-1 in pneumokokken meningitis anders is dan de inhibitie van leukocyten influx. Verder vonden we twee MMP's geassocieerd met ziekte uitkomst. In de laatste jaren is steeds meer bekend geworden over de rol van MMP's als immuno-modulerende proteases, naast hun bekende functie als modulators van de extracellulaire matrix.<sup>48</sup> Er is nog weinig bekend over de rol van MMP-1 of MMP-3 in pneumokokken infecties. Onder de bekende substraten van MMP-1 vallen onder andere meerdere inflammatoire mediators zoals pro-TNF, IL-1 $\beta$  en meerdere chemokines waarvan bekend is dat sommigen in receptor antagonist worden gekleefd en dus een anti-inflammatoir effect hebben.<sup>49</sup> IL-10 en IL-1RA zijn anti-inflammatoire cytokines, die betrokken zijn bij het beperken van weefselschade als gevolg van een infectie.<sup>50</sup> IL-10 concentraties in onze studie zijn sterk gecorreleerd aan IL-1RA concentraties. IL-1RA is een antagonist van de IL-1 receptor, waardoor binding van IL-1 $\alpha$  en IL-1 $\beta$  wordt bemoeilijkt. In onze studie zijn IL-1RA/IL-1 $\beta$  ratio's fors lager dan de levels die volgens de literatuur nodig zijn om IL-1 $\beta$  effectief te antagoneren. Dus de IL-1RA concentraties zijn of een ontoereikende poging om IL-1R signalering te antagoneren of IL-1RA heeft een andere nog onbekende functie naast het antagoneren van de IL-1R. Recent is beschreven dat IL-1RA in staat is NLRP3 signalering te inhiberen.<sup>51</sup> Het was onverwacht dat we twee anti-inflammatoire mediators vonden die geassocieerd waren met slechte uitkomst. Mogelijk duidt dit op een falende onderdrukking van de pro-inflammatoire respons, of een overactieve anti-inflammatoire respons zou ook kunnen leiden tot ineffektieve controle van de infectie en bestrijden van de bacteriën, en daardoor leiden tot slechte ziekte uitkomst.

CCL7 en CXCL5 zijn beiden chemo-attractanten van neutrofielen, en daarmee pro-inflammatoir. In een pneumokokken pneumonie model had CCL7 een pro-inflammatoir effect.<sup>52</sup> Echter, CCL7 kan ook worden gemoduleerd in een receptor antagonist, resulterend in een anti-inflammatoir effect. De assay die wij hebben gebruikt maakt geen onderscheid tussen de pro- en anti-inflammatoire variant van CCL7. In eerdere studies is aangetoond dat CXCL5 is verhoogd in de liquor van patiënten met bacteriële meningitis en dat het chemo-attractief is voor neutrofielen.<sup>53</sup>

Limitaties van onze studie zijn onder andere een inclusie bias (alleen patiënten met een positieve liquor kweek zijn geïnccludeerd, waardoor ernstig patiënten met een contra-indicatie voor een lumbaalpunctie zijn geëxcludeerd), verder zijn er verschillen in de behandeling van de samples in de verschillende ziekenhuizen. Uit deze studie kunnen geen conclusies worden getrokken of de gevonden inflammatoire mediators intrathecally worden geproduceerd of de bloed-hersen barrière passeren vanuit het systemische compartiment, of hoe deze levels zich verhouden tot de bacterie load in de liquor. Voor toekomstige experimenten waarbij inflammatoire mediators worden geanalyseerd in de liquor zou het interessant zijn om dit te combineren met gepaarde bloed samples, en de bacteriële load in de liquor te meten. Verder kunnen geen conclusies uit deze studie worden getrokken ten aanzien van een causale relatie tussen de inflammatoire mediators en de ziekte uitkomst. Hiervoor zijn vervolgstudies nodig naar de rol van deze inflammatoire mediators in de pathofysiologie van pneumokokken meningitis.

## Toekomstig onderzoek

Toekomstig onderzoek naar pneumokokken meningitis zal zich vooral richten op het hypothese vrij vinden van nieuwe pathways en biologische processen die betrokken zijn bij de ziekte uitkomst. In het verlengde hiervan worden klinische patiënt data, genetische patiënt data, bacteriële genetische data en liquor inflammatoire mediators en hun onderlinge relatie met elkaar geanalyseerd. Als onderdeel van de MeninGene studie is DNA verzameld van een groot cohort patiënten, en in 198 gevallen zijn zowel liquor samples als DNA van de patiënt beschikbaar voor analyse, en dit aantal groeit nog steeds. In deze patiënten is een grote set exoom varianten bepaald, en deze data worden momenteel gecorreleerd aan de concentraties inflammatoire mediators in de liquor. In alle gevallen is de verwekker geïsoleerd en opgeslagen. Genetische analyse van de geïsoleerde *S. pneumoniae* stam in combinatie met de patiënten data zal leiden tot nieuwe inzichten in de host-pathogeen interactie. Deze hypothese vrije aanpak zal hopelijk leiden tot de identificatie van nieuwe eiwitten, pathways of processen die betrokken zijn in de pathofysiologie van pneumokokken meningitis.

Ten tweede, is het interessant om het onderzoek naar de gevonden inflammatoire mediators in hoofdstuk 7 verder voort te zetten. Daarbij zou dan eerst gekeken moeten worden of deze inflammatoire mediators tot expressie komen in ons pneumokokken muis model, en zo ja in welke cel typen, en of deze inflammatoire mediators betrokken, of slechts markers zijn van een slechte ziekte uitkomst. Deze laatste vraag kan worden beantwoord door bijvoorbeeld een vergelijkend onderzoek te doen met knock out muizen in ons pneumokokken meningitis model. Vervolgens zou het interessant zijn om te kijken of de inflammatoire mediators een agonist of antagonistische werking hebben, en om meer inzicht te krijgen in hun functie in de immuun respons. Omdat we twee anti-inflammatoire cytokines geassocieerd met uitkomst vonden (IL-10 en IL-1RA), spelen

mogelijk regulatoire T-cellen een rol, waarvan bekend is dat ze de immuun respons in bedwang houden. In de laatste jaren is duidelijk geworden dat in ouderen, met een hoge incidentie pneumokokken meningitis, regulatoire T-cellen actiever zijn, en dus een groter immunosuppressief effect hebben.<sup>54,55</sup> Om meer inzicht te krijgen in de mate van controle van de bacteriële groei gedurende de infectie in relatie met de inflammatoire mediators die zijn gevonden in de liquor, en of deze inflammatoire mediators geproduceerd worden in het centraal zenuwstelsel of in het systemische compartiment, zou data moeten worden verzameld ten aanzien van de bacteriële load in de liquor en concentraties van inflammatoire mediators in het bloed in ons patiënt cohort.

Concluderend, hebben we aangetoond dat inflammasome signalering geassocieerd is met systemische complicaties, en dat inflammasome onafhankelijke NLRP3 signalering hersenschade induceert in ons pneumokokken meningitis muis model. We vonden dat virulentie factoren van de pneumokok een rol spelen in de klinische uitkomst van de patiënt. Afwezigheid van een arginine synthetase systeem in de geïsoleerde pneumokokken stam was geassocieerd met goede uitkomst in deze patiënten. Ten slotte, hebben we een aantal nieuwe inflammatoire mediators geassocieerd met ziekte uitkomst, waaronder twee MMP's, twee anti-inflammatoire cytokines, twee chemokines en een adhesie molecuul, wat suggereert dat deze betrokken zijn bij de pathofysiologie van pneumokokken meningitis. Vervolgonderzoek zal zich richten op het combineren van klinische en genetische patiënt data, genetische data van de geïsoleerde *S. pneumoniae* stam, en de concentratie inflammatoire mediators in de liquor, om zo meer inzicht te krijgen in de gastheer-pathogeen interactie, en het effect op de inflammatoire respons en ziekte uitkomst van de patiënt. We hopen dat dit leidt tot de identificatie van nieuwe pathways of biologische processen die betrokken zijn bij de ziekte uitkomst van pneumokokken meningitis, die dan vervolgens kunnen worden onderzocht in ons meningitis muis model om zo nieuwe potentiële behandelingen te vinden die ons uiteindelijk terugbrengen van de laboratorium tafel naar de patiënt.



## LIST OF ABBREVIATIONS

## List of abbreviations:

Adr: acetyltransferase  
AIM2: absent in melanoma 2  
APC: antigen presenting cell  
ArgG: argininosuccinate locus  
ArgH: argininosuccinate lyase locus  
ASC: apoptosis-associated speck-like protein  
C1q: complement factor 1q  
CARD: caspase activation and recruitment domain  
CBF: cerebral blood flow  
Cbpa: choline-binding protein A  
CFU: colony forming units  
CI: confidence interval  
CNS: central nervous system  
CRP: C-reactive protein  
CSF: cerebrospinal fluid  
ECM: extracellular matrix  
ENA-78: epithelial derived neutrophil activating peptide-78  
ERK: extracellular signal-regulated kinase  
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase  
GCS: Glasgow Coma Score  
G-CSF: granulocyte-colony stimulating factor  
GM-CSF: granulocyte macrophage-colony stimulating factor  
GOS: Glasgow Outcome Scale  
ICAM-1: soluble intercellular adhesion molecule  
ICP: intracranial pressure  
IFN: interferon  
IL-1RA: interleukin-1 receptor antagonist  
IL: interleukin  
IQR: interquartile range  
KC: keratinocyte-derived chemokine  
LDA: linear determinant analysis  
LFA: lymphocyte function-associated antigen  
LLOQ: lower limit of quantification  
LPS: lipopolysaccharide  
LTA: lipoteichoic acid  
MBL: mannose binding lectin  
MCP: monocyte chemoattractant protein  
M-CSF: macrophage colony stimulating factor  
MIF: macrophage migration inhibitory factor

MIP: macrophage inflammatory protein  
MMP: matrix metalloproteinase  
MPO: mouse myeloperoxidase  
NF- $\kappa$ B: nuclear factor kappa B  
NLR: Nod-like receptor  
NLRC: Nod-like receptor family, CARD containing  
NLRP: Nod-like receptor family, pyrin domain containing  
Nod: nucleotide oligomerization domain  
OR: odds ratio  
PAF: platelet activating factor  
PAI: plasminogen activator inhibitor  
PAMP: pathogen associated molecular pattern  
PavA: pneumococcal adhesion and virulence protein A  
PCW: pneumococcal cell wall  
PIgR receptor: polymeric immunoglobulin receptor  
Ply: pneumolysin  
PspA: pneumococcal surface protein A  
PspC: pneumococcal surface protein C  
RNS: reactive nitrogen species  
ROS: reactive oxygen species  
sCD40L: soluble cluster of differentiation 40 ligand  
SCF: stem cell factor  
sIgA: secretory IgA  
TACE: TNF- $\alpha$  converting enzyme  
TAFI: thrombin activatable  
TF: tissue factor fibrinolysis inhibitor  
TFPI: tissue factor pathway inhibitor  
TGF: transforming growth factor  
TLR: Toll-like receptor  
TNF: tumor necrosis factor  
tPA: tissue-type plasminogen activator  
Treg: regulatory T-cells  
ULOQ: upper limit of quantification  
uPA: urokinase-type plasminogen activator  
VCAM-1: soluble vascular cell adhesion molecule-1  
VEGF: vascular endothelial growth factor  
WT: wild-type



DANKWOORD

## Dankwoord

Dit proefschrift is tot stand gekomen door de samenwerking van veel verschillende mensen. Graag wil ik iedereen bedanken die heeft bijgedragen, zonder jullie was dit proefschrift er niet geweest. Een aantal mensen wil ik in het bijzonder bedanken.

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Promotor prof. dr. Tom van der Poll. Beste Tom, door jou enorme kennis en ervaring op het gebied van experimenteel onderzoek naar infectieziekten is het gelukt om ons meningitis model snel en efficiënt lopende te krijgen. Maar ook daarna, toen bleek dat onze resultaten niet altijd even eenduidig waren, wist jij daar de juiste sturing aan te geven. Onze gesprekken over welke kant we op moesten met ons onderzoek waren voor mij altijd erg verhelderend. Je gaf vaak geen direct advies wat we moesten doen, maar door de lijn in de discussie liet je ons zelf tot de juiste conclusies komen. Ik heb veel van je geleerd op wetenschappelijk gebied. Je positieve en toch kritische reacties op de (vervolgens afgewezen) manuscripten werkten enorm motiverend. En de infectieuze partijtjes waren groot feest!

Co-promotor dr. Matthijs Brouwer. Beste Matthijs, initieel werkten we veel aan inclusies in de databases en de analyses van de liquor samples, en later werd je mijn copromotor. Je snelle werken en scherpe blik op de manuscripten waren erg fijn (en soms ook frustrerend als je toch nog een typo wist te vinden in een poster die inmiddels al bij de drukker lag). Door je uitgebreide kennis van cohort- en genetisch onderzoek heb ik veel van je geleerd. Je voorliefde voor de foute humor van onder andere de Mannen van de Radio en Radio Bergeijk zorgden voor de dagelijkse dosis gezonde humor op de meningitis kamer. Bedankt voor je begeleiding!

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# CURRICULUM VITAE

## Curriculum vitae

Madelijn Geldhoff was born on April 22<sup>nd</sup> 1980, in Lienden, the Netherlands. She graduated from secondary school (Christelijk Lyceum Veenendaal) in 1999. In the same year she started a study on Biomedical Sciences at Utrecht University and in 2001 she started Medical School. During her studies she participated as a student member in the Educational Committee of the faculty of Biomedical Sciences of Utrecht University from 2000 to 2002. Along the way she got interested in both neurology and infectious diseases and did a 6-month research internship on temporal lobe epilepsy at the Brain Center Rudolph Magnus in Utrecht. In 2007 she started a 9-month research internship at the Department of Infectious Diseases at Children's Hospital, Harvard University, Boston (USA) focusing on an intranasal pneumococcal vaccine. In 2007 she earned her Doctor of Medicine degree and in 2008 a Master's degree in Biomedical Sciences.

In 2008 she worked as a resident not in training at the Intensive Care Unit of the Diakonessen Hospital Utrecht, and in 2009 she started as a PhD candidate at the Department of Neurology of the Academic Medical Center (AMC), Amsterdam (under supervision of prof. dr. D. van de Beek and prof. dr. T. van der Poll) which lead to the publication of this thesis. In 2012 she started her residency in Neurology at the AMC under supervision of prof. dr. I.N. van Schaik, prof. dr. J. Stam and dr. J.H.T. Koelman. Currently she is situated at the OLVG Oost under supervision of dr. V.I.H. Kwa.

Madelijn lives together with Jan Willem van den Besselaar, their daughter Miep and son Brecht, and are expecting a second son in November 2016.

## LIST OF PUBLICATIONS

## List of publications

1. **Geldhoff M**, Mook-Kanamori BB, van de Beek D. Macrophage migration inhibitory factor, infection, the brain, and corticosteroids. *Crit Care* 2009;13:170.
2. Mook-Kanamori B, **Geldhoff M**, Troost D, van der Poll T, van de Beek D. Characterization of a pneumococcal meningitis mouse model. *BMC Infect Dis* 2012;12:71.
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5. **Geldhoff M**, Mook-Kanamori BB, Brouwer MC, Troost D, Leemans JC, Flavell RA, Van der Ende A, Van der Poll T, Van de Beek D. Inflammasome activation mediates inflammation and outcome in humans and mice with pneumococcal meningitis. *BMC Infect Dis* 2013;13:358.
6. Piet JR, **Geldhoff M**, van Schaik BD, Brouwer MC, Valls Seron M, Jakobs ME, Schipper K, Pannekoek Y, Zwinderman AH, van der Poll T, van Kampen AH, Baas F, van der Ende A, van de Beek D. *J Infect Dis* 2014;209:1781-91.
7. Adriani KS, Brouwer MC, **Geldhoff M**, Baas F, Zwinderman AH, Paul Morgan B, Harris CL, van der Ende A, van de Beek D. Common polymorphisms in the complement system and susceptibility to bacterial meningitis. *J Infect* 2013;66:255-62.
8. Mook-Kanamori BB, Brouwer MC, **Geldhoff M**, Ende A, van de Beek D. Cerebrospinal fluid complement activation in patients with pneumococcal and meningococcal meningitis. *J Infect* 2014;68:542-7.
9. Koopmans MM, Brouwer MC, **Geldhoff M**, Seron MV, Houben J, van der Ende A, van de Beek D. Cerebrospinal fluid inflammatory markers in patients with *Listeria monocytogenes* meningitis. *BBA Clin* 2014;1:44-51.
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11. Valls Serón M, Duitman J, **Geldhoff M**, Engelen-Lee J, Havik SR, Brouwer MC, van de Beek D, Spek CA. CCAAT/enhancer-binding protein delta (C/EBPdelta) aggravates inflammation and bacterial dissemination during pneumococcal meningitis. *J Neuroinflammation* 2015;12:88.
12. Mook-Kanamori BB, Valls Serón M, **Geldhoff M**, Havik SR, van der Ende A, Baas F, van der Poll T, Meijers JC, P Morgan B, Brouwer MC, van de Beek D. Thrombin-activatable fibrinolysis inhibitor influences disease severity in humans and mice with pneumococcal meningitis. *J Thromb Haemost* 2015;13:2076-86.
13. Ferwerda B, Valls Serón M, Jongejan A, Zwinderman AH, **Geldhoff M**, van der Ende A, Baas F, Brouwer MC, van de Beek D. Variation of 46 Innate Immune Genes Evaluated for their Contribution in Pneumococcal Meningitis Susceptibility and Outcome. *EBioMedicine* 2016.
14. Valls Serón M, Ferwerda B, Engelen-Lee J, **Geldhoff M**, Jaspers V, Zwinderman AH, Tanck MW, Baas F, van der Ende A, Brouwer MC, van de Beek D. V-akt murine thymoma viral oncogene homolog 3 (AKT3) contributes to poor disease outcome in humans and mice with pneumococcal meningitis. *Acta Neuropathol Commun* 2016;4:50.

## Research portfolio

Name PhD student: M. Geldhoff

PhD period: 2009 - 2016

Name PhD supervisor: prof. dr. van de Beek

<b>PhD training</b>	year	Workload (ECTS)
<b>General courses</b>		
Graduate School course Infectious Diseases	2010	1.3
Graduate School course on clinical epidemiology	2011	0.9
Graduate School course practical biostatistics	2011	1.1
<b>Specific courses</b>		
ESCMID postgraduate technical workshop histopathology in experimental neuroinfection	2009	1
<b>Seminars, workshops and masterclasses</b>		
Masterclass by Charles Dinarello, 2011, Amsterdam, The Netherlands	2011	0.5
Weekly research meetings of the Center of Experimental and Molecular Medicine (CEMM), Academic Medical Center Amsterdam, The Netherlands	2009-2011	5.6
Weekly journal club of the Center of Experimental and Molecular Medicine (CEMM), Academic Medical Center Amsterdam, The Netherlands	2009-2011	5.6
Two-weekly research meetings, Department of Neurology, Academic Medical Center Amsterdam	2009-2016	6.5
Monthly research meetings of the Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center Amsterdam, The Netherlands	2009-2011	1.5

<b>PhD training</b>	year	Workload (ECTS)
<b>Presentations</b>		
The inflammasome in experimental pneumococcal meningitis, poster presentation, ICAAC 2010, Boston, United States	2010	0.5
The inflammasome, oral presentation, 2-weekly research meetings, Department of Neurology, Academic Medical Center Amsterdam	2011	0.5
The inflammasome in experimental pneumococcal meningitis, oral presentation, Masterclass Charles Dinarello, Amsterdam The Netherlands	2011	0.5
Cerebrospinal fluid chemokine levels and outcome in bacterial meningitis, poster presentation 51th ICAAC, Chicago, United States	2011	0.5
The inflammasome in experimental pneumococcal meningitis, poster presentation TOLL2011 meeting	2011	0.5
Cerebrospinal fluid inflammatory mediators in patients with pneumococcal meningitis, oral presentation ECCMID 2016, Amsterdam, The Netherlands	2016	0.5
<b>International conferences</b>		
47 <sup>th</sup> annual meeting of the Infectious Diseases Society of America (IDSA), Philadelphia, United states	2009	1.25
50 <sup>th</sup> Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Boston, United States	2010	1.25
TOLL 2011 meeting, Riva del Garda, Italy	2011	1.25
26 <sup>th</sup> annual meeting of the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2016, Amsterdam, The Netherlands	2016	1.25
<b>Parameters of Esteem</b>		
<b>Grants</b>		
Student travel grant ICAAC 2010, Boston United States	2010	





