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ORIGINAL PAPER

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)-1β 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.

Keywords Bacterial · Meningitis · Inflammasome · Inflammation · Genetic Association Study · Infectious disease

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Introduction

Bacterial meningitis is associated with high mortality, even in developed countries despite the implementation of childhood vaccination programs and effective antimicrobial agents (van de Beek et al. 2004, 2006). The most common causative agent is *Streptococcus pneumoniae*, with case fatality rates ranging from 16 to 37 % (van de Beek et al. 2004, 2006; Weisfelt et al. 2006), and neurological sequelae, including hearing loss (Heckenberg et al. 2012), focal neurological deficits, and cognitive impairment, occurring in 30–52 % of surviving patients (Hoogman et al. 2007; van de Beek et al. 2002). Host genetic variation has been shown to influence susceptibility and outcome of pneumococcal and meningococcal infections (Brouwer et al. 2009; Brouwer et al. 2010).

The inflammasomes are intracellular signaling complexes belonging to the Nod-like receptors (NLRs) (Lamkanfi and Dixit 2011; Schroder et al. 2010; Stutz et al. 2009). To date, four major inflammasome complexes have been described, of which the Nod-like receptor protein 3 (NLRP3) inflammasome has been investigated most extensively (Lamkanfi and

Dixit 2011; Schroder et al. 2010; Stutz et al. 2009). The inflammasomes can be activated by several endogenous as well as exogenous danger signals, including ATP, changes in K^+ concentration, oxygen radicals, and uric acid released through cell injury in inflammation (Franchi et al. 2009; Rathinam et al. 2012). 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 (Franchi et al. 2009; Rathinam et al. 2012). 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-1β) and pro-IL-18 into the respective active and secreted cytokines (Lamkanfi and Dixit 2011; Schroder et al. 2010; Stutz et al. 2009).

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-1β and IL-18 production (Razmara et al. 2002).

Several findings in patients and animal models suggest a pivotal role for inflammasomes in the pathophysiology of bacterial meningitis (Mook-Kanamori et al. 2011). Firstly, in adults with bacterial meningitis, cerebrospinal fluid (CSF) levels of caspase-1 were elevated compared to noninfected patients (Koedel et al. 2002). Furthermore, in children with pneumococcal meningitis, IL-1β concentrations in the CSF were elevated, a finding that also has been observed in various animal models (Barichello et al. 2010; Saukkonen et al. 1990; Schmidt et al. 1999). While the role of IL-1β in the pathogenesis of pneumococcal meningitis has not been elucidated yet, various clinical effects have been attributed to caspase-1, IL-1β, and IL-18-mediated processes (Hoegen et al. 2011; Koedel et al. 2002; Mook-Kanamori et al. 2011; Saukkonen et al. 1990). 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 (Hoegen et al. 2011).

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-1β 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 (Jennett et al. 1976). 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*, *NLRC4*, *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 (Roberts et al. 2010). No assays for common nonsynonymous SNPs in the coding regions of *NLRC4* were commercially available. Laboratory personnel were blinded to clinical information.

IL-1β and IL-18 measurements in CSF samples of patients with bacterial meningitis

We measured IL-1β 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 °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 °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 χ^2 test. These statistical tests were two-tailed, and $P<0.05$ was regarded as significant. Differences in genotype frequencies were analyzed with the χ^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 χ^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 rs2043211genotype to assess if a gender specific association of this SNP influenced outcome (Schoultz et al. 2009).

Results

A total of 801 Dutch patients with bacterial meningitis were included as described previously (Woehrl et al. 2011). 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. 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.

Table 1 Clinical characteristics of 531 patients with community acquired bacterial meningitis [data are number/number evaluated (percentage) or mean±SD]

Characteristic	Value/total	Characteristic	Value/total
Age (years)	55±17	Indexes of CSF inflammation[b]	
Male sex	262 (49 %)	Opening pressure (mmH_2O)	34±11
Pretreatment with antibiotics	63/527 (12 %)	WBC ($/mm^3$)	6778±13319
Predisposing conditions	227 (43 %)	WBC < $1{,}000/mm^3$	142/496 (27 %)
Otitis or sinusitis	191 (36 %)	Protein (g/l)	4.3±3.0
Pneumonia	77 (15 %)	CSF blood: glucose ratio	0.14±0.19
Immunocompromise	124 (23 %)	Positive blood cultures	346/463 (75 %)
Symptoms and signs on presentation		Complications	
Headache	411/479 (85 %)	Systemic complications	166 (31 %)
Neck stiffness	398/510 (78 %)	Neurological complications	327 (62 %)
Systolic blood pressure (mmHg)	146±29	Glasgow Outcome Scale	
Heart rate (bpm)	99±21	1—Death	40/528 (8 %)
Body temperature (°C)	38.7±1.3	2—Vegetative state	1/528 (0.2 %)
Score on Glasgow Coma Scale[a]	11±3	3—Severe disability	21/528 (4 %)
<8 indicating coma	70/527 (13 %)	4—Moderate disability	78/528 (15 %)
Focal neurological deficits	141/528 (27 %)	5—Good recovery	388/528 (73 %)

[a] Glasgow coma scale score was evaluated in 527 patients

[b] CSF pressure was evaluated in 123 patients, CSF WBC in 496, CSF protein in 505, and CSF blood to glucose ratio in 498

CSF was obtained from 289 patients with bacterial meningitis and 19 control patients. Levels of IL-1β 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-1β 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-1β 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) 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-1β and IL-18 between patients were not associated with rs2043211 and rs11621270 genotypes, also after a correction for total CSF protein levels was applied.

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

Gene	SNP	Good outcome (GOS 5)[a]					Poor outcome (GOS 1–4)					Model	*P* value
	ID	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

[a] Glasgow Outcome Scale Score

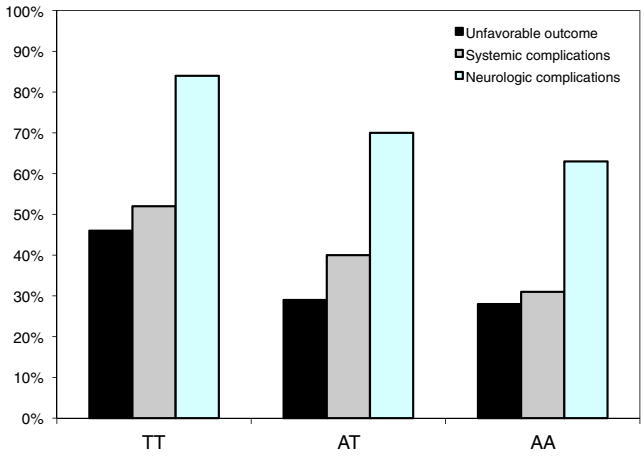

Fig. 1 Rate of mortality, systemic, and neurological complications by *CARD8* rs2043211 genotype in pneumococcal meningitis patients. *CARD8* rs2043211 was associated with poor outcome an additive model (P=0.040). Patients with the T/T genotype had the highest risk for poor outcome [odds ratio (OR), 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)

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-1β 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 (Kastbom et al. 2010; McGovern et al. 2006; Roberts et al. 2010; Schoultz et al. 2009; Yang et al. 2011). Several functions have been attributed to CARD8. First is the inhibition of pathways to nuclear factor kappa B (NF-κB) activation (Bouchier-Hayes et al. 2001); (Fontalba et al. 2007). In vitro studies have demonstrated that CARD8 interferes with NF-κB activation by established NF-κB activators, possibly through direct interaction between CARD8 and IkB kinase complex (Bouchier-Hayes et al. 2001). Second, CARD8 also has anti-apoptotic 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) (Checinska et al. 2006). Through direct interaction with caspase-1, CARD8 can negatively regulate caspase-1 dependent IL-1β generation in vitro (Razmara et al. 2002). Lastly, CARD8 forms a physical component of the multiprotein complex of the NLRP3 inflammasome (Agostini et al. 2004; Dinarello 2004). 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 (Allen et al. 2009).

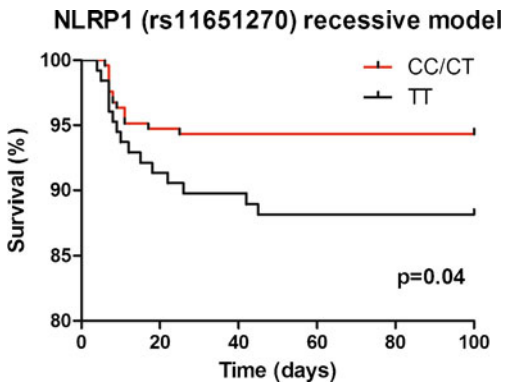

Fig. 2 Kaplan–Meier survival curve in patients with pneumococcal meningitis according to rs11651270 genotype

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-κB mediated proinflammatory (pro)cytokines, incomplete NLRP3 assembly, and a limited caspase-1 activation, resulting in limited secretion of activated IL-1β and IL-18. Conversely, the normal form of CARD8 (T/T genotype) may lead to NF-κB inhibition and lower proinflammatory (pro)cytokines. Despite proper NLRP3 assembly with CARD8, this could *also* lead to limited secretion of IL-1β and IL-18. Indeed, we do not see a difference in CSF levels of IL-1β 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

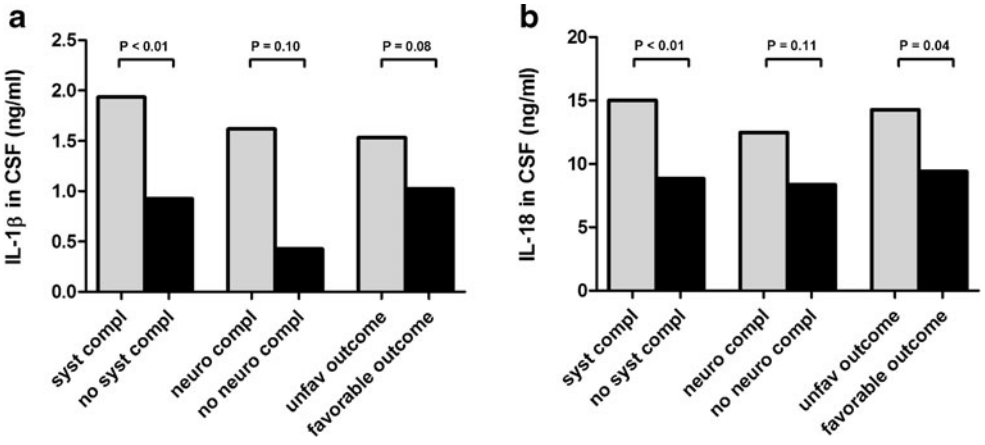

Fig. 3 Median levels of IL-1β and IL-18 in CSF of patients with bacterial meningitis. **a** Elevated IL-1β was associated with systemic complications, and there was trend towards more neurological complications. **b** High IL-18 levels were associated with both systemic complications and unfavorable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

disease, type 1 diabetes, and Alzheimer's disease (Franchi et al. 2009; Jin et al. 2007; Magitta et al. 2009). 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 and myramyl dipeptide, a peptidoglycan constituent of both Gram-positive and Gram-negative bacteria (Dowling and O'Neill 2012). 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) (Rathinam et al. 2012). However, as the rs11651270 polymorphism does not seem to influence levels of IL-1β 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 (D'Osualdo et al. 2011). Interestingly, the aforementioned NLRP1 SNP lies *in*, and the CARD8 SNP is situated *before* the respective FIIND regions (D'Osualdo et al. 2011). 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-1β 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-1β and IL-18 are also being produced in an inflammasome-independent manner. This was previously shown for IL-1β, which can be produced by neutrophil-derived serine proteases or pathogen-derived proteases (Netea et al. 2010). Therefore, a small potential decrease in cytokine production due to *NLRP1* and *CARD8* polymorphisms may not be measurable in the total amount of IL-1β and IL-18 produced. Another explanation may be that the impact of these polymorphisms on secreted active IL-1β and IL-18 levels is limited, and *NLRP1* and *CARD8* may be involved in alternative inflammatory roles (Netea et al. 2010). 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.

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