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DISCUSSION

From the first description by Vieusseux in 1806 until the early twentieth century, acute bacterial meningitis (BM) was quite uniformly considered a fatal disease. Despite the introduction of antibiotics, introduction of universal immunization strategies, and continuing improvement of supportive care, mortality remains unacceptably high. Although the incidence is decreasing in developed countries, it is still one of the top ten infectious causes of death worldwide. We have discovered specific genes involved in the susceptibility, severity and the risk for development of hearing loss after this disease by studying variation in host innate immune response genes in children who survived BM.

In this thesis it was shown for the first time that polymorphisms in Toll-like receptors -4, -9 and Nucleotide oligomerization domain protein (NOD)-2 are associated with susceptibility to BM during childhood. This may help to explain why severe invasive disease and meningeal inflammation occurs in a restricted number of people while a majority of people are asymptomatic carriers. TLR4 (combined with TLR2 or TLR9) was also associated with post-meningitis hearing loss. TLR9 SNPs and haplotypes are associated with severity in the acute phase of meningitis; the -1237 “C” variant allele was associated with protection against bacteremia, increased CSF leukocytes levels and decreased CSF/blood glucose ratios. Furthermore, a pre-existing prediction model for post-meningitis hearing loss was validated in an external cohort, while another model for academic and behavioral limitations could not be validated. Addition of these former high-risk genes to the prediction model for post-meningitis hearing loss did not significantly improve the model and requires specific improvements. Integration of high-risk SNPs in clinical prediction models is a promising new concept and may provide an important basis for (ongoing) future studies. These results will be discussed later in detail after some background information about genetic association studies.

Genetic association studies in general

Although humans are identical at most of the 3 billion base pairs in their genome, inter-individual variation is present in approximately 0.1% of the genome. SNPs, common variants with a frequency of more than 1% in a population, are the most common type of human genetic variation. SNPs may alter the aminoacid sequence (non-synonymous SNPs), affect promoter characteristics or may be completely ‘silent’ (Figure 1). SNPs occur normally throughout a person’s DNA approximately once in every 300 nucleotides. Although some SNPs have been identified with important clinical consequences, most SNPs are thought to have no or only a minor contribution to the phenotype. The simultaneous evaluation of epistatic interactions among multiple SNPs may provide
opportunities to detect contributions of small-effect loci to complex diseases risk\(^5\). In this thesis the potential importance of gene-gene interactions in SNP association was studied. This concept has been used in only a few other studies on complex diseases\(^6\),\(^7\).

**FIGURE 1.** Schematic representation of a non-synonymous, functional SNP. A single change in a base pair sequence may lead to altered protein configuration and altered binding of a receptor to a ligand. SNPs in a non-coding region may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA.
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Selection of genes
In order to select genes, the candidate gene approach was used, which is a method to investigate the validity of an “educated guess” about the genetic basis of a disorder. In detail, the first step is selection of an allele (or a set of alleles) in a gene or its promoter based on a hypothesis using literature, associations of the SNP with other diseases or functional studies. This gene is called a candidate gene. Studies using knock-out mice, for example, provide important evidence for the relevance of specific genes in BM. The next step is to identify variants in or near those genes that might either cause a change in the function of a protein or its expression, or be in linkage with other alleles encoding for peptides involved in functional changes. The last step is to test these SNPs by comparing genotype distributions between affected and unaffected subjects. Biological mechanisms explaining significant associations can further be studied for example by mRNA expression profiling or comparison of protein levels in cases and controls.

TLR4 is associated with susceptibility to meningitis
Highly variable individual responses to lipopolysaccharides (LPS) are observed and may be due to genetic variation in TLR4. Arbour et al. were the first to report that humans with a TLR4+896 A>G (Asp>Gly) missense mutation affecting the extracellular domain of the TLR4 receptor showed hyporesponsiveness to inhaled LPS in vitro, which was restored after transfection with the wild type allele of TLR4. This initiated many genetic association studies with conflicting results. In contrast to our findings, in Gambian children with serogroup A meningococcal meningitis (MM) no association was found with TLR4 +896 and susceptibility to MM. This may be due to the fact that 86% of our Dutch Caucasian cohort consisted of serogroup B patients with only one case of serogroup A. In our study, carriers of TLR4 +896 mutations are more susceptible to MM, which is in line with other studies on susceptibility to invasive meningococcal disease. The proposed mechanism is that carriers of the TLR4 mutant are hyporesponsive to LPS and therefore exhibit a decreased inflammatory response upon meningococcal infection. This mechanism may be similar in the CNS since TLR4 is widely present on CNS resident cells after infection.

TLR9 is associated with susceptibility and severity of meningitis
The TLR9+2848 mutant was associated with protection against BM while haplotype 1 (lacking both TLR9 mutant alleles) increases the risk for BM and meningococccemia. It is tempting to speculate that the TLR9 -1237 C mutant results in an increased immune response upon activation by N. meningitidis. This hypothesis is in line with the associations of this allele with protection against MM and bacteremia, higher CSF leukocytes and altered CSF blood/glucose ratios.
DISCUSSION

Since TLR9 is an intracellular receptor, phagocytosis of meningococci must occur before binding of CpG to TLR9 and initiation of the immune response may take place. Phagocytosis of meningococci occurs during transcellular passage of these bacteria through TLR9 expressing sinonasal epithelial cells or in neutrophils in the circulation during complement-mediated opsonophagocytosis. Inside the CNS meningococci are recognized by astrocytes, microglia, dendritic cells and macrophages. CSF from patients with MM reveals bacteria attached to and inside neutrophils. Intracellular meningococcal DNA motifs activate endosomal TLR9, initiating signal transduction with subsequent production of cytokines and chemokines, leading to leukocyte recruitment towards the CNS. A possible biological explanation for increased immune responses in TLR9-1237 C carriers is increased NFκB binding to the TLR9 promoter region, leading to increased transcriptional regulation of TLR9. Convincing evidence for this hypothesis was provided in several studies. First, we and others predicted with *In silico* analysis that the -1237 C allele creates several novel binding sites for different transcription factors including NFκB. Using a luciferase reporter assays and Noshift transcriptional factor (DNA-protein) interaction analysis, Ng *et al.* confirmed that TLR9 transcriptional activity of the C allele is consistently higher than the wildtype when innate immunity pathways are activated and this is due to increased binding affinity to NFκB. Carvalho *et al.* reported that the TLR9-1237 C allele introduced a new IL-6-dependent transcription factor binding site in the TLR9 promoter. Peripheral blood mononuclear cells (PBMCs) harbouring the TC genotype show higher expression of TLR9 and IL-6 in response to CpG. Another study showed higher serum interferon-γ levels in children with cerebral malaria carrying the TLR9-1237-C, indicating that enhanced TLR9 mediated immune responses are also relevant inside the CNS. The TLR9+2848 SNP does neither result in an amino acid change nor in the modification of a regulatory site, implying linkage of a functional relevant SNP in the vicinity of this SNP, underscored by the finding that haplotype 1 (lacking both TLR9 mutant alleles) increases the risk for BM.

*In silico analysis*

*In silico* analysis of pathogen genomes showed a very strong immune inhibitory potential for *N. meningitidis* CpG DNA, while *S. pneumoniae* DNA showed a mildly immunostimulatory potential. High concentrations of bacterial DNA are required and higher genomic frequencies of CG dinucleotides result in increased activity of TLR9. The ratio between immune stimulatory and inhibitory sequences affects the ability of CpG to activate antigen presenting cells via TLR9. TLR9 SNPs may result in an increased activation by meningococcal CpG, abolishing its strong immune-inhibitory effect, leading to increased immune activation. There is mounting evidence that *N. meningitidis* manipulates and evades the innate immune response to promote infectious processes. A good example is...
the ability of \textit{N. meningitidis} to avoid phagocytosis by neutrophils by preventing binding to the neutrophil surface.\textsuperscript{23} The \textit{TLR9} mutant may impede these mechanisms.

\textit{The potential role of TLR9 SNPs in pathogenesis of meningococcal meningitis}

The importance of TLR9 in host defense against meningococcemia is well illustrated by the observation that mice with meningococcal sepsis displayed reduced survival and elevated levels of bacteremia compared to wildtype mice\textsuperscript{21}. We found an association between \textit{TLR9} SNPs and decreased incidence of bacteremia and higher leukocyte levels in the CSF. The link between systemic and meningeal infection has been previously studied. Darton \textit{et al.} found no correlation between bacterial loads in blood and CSF in 239 patients with serogroup B MM. Extremely high CSF bacterial loads were observed despite modest levels of bacteremia\textsuperscript{31}. In another study most severely affected patients with MM or sepsis with rapidly evolving septic shock and high mortality had significant lower levels of pleocytosis\textsuperscript{32}. Injecting rabbits with systemic- prior to intracisternal- LPS led to impaired pleocytosis and reduced levels of tumor necrosis factor (TNF), reflecting impaired inflammatory responses in the CNS\textsuperscript{33}. The proposed mechanism is that bacteremia leads to a decrease in numbers of peripheral leucocytes, reducing CSF pleocytosis. \textit{TLR9} -1237 and +2848 polymorphisms have a beneficial effect on preventing bacteremia but increase the leukocyte influx into the CNS, which may reflect an enhanced immune response inside the CNS. Decreased CSF/blood glucose ratios observed in \textit{TLR9} C carriers may reflect higher glucose consumption by immune active cells in the CNS.

Future studies on the functional consequences of \textit{TLR9} SNPs in BM should include studies comparing TLR9 expression and immune activation in whole blood of \textit{TLR9}-1237 wildtype carriers with mutant carriers before and after stimulation with live \textit{N. meningitidis} and \textit{S. pneumoniae}. Increased TLR9-expression and cytokine levels after stimulation in C variant carriers will be in line with our findings that TLR9 transcription and function is upregulated in mutant carriers. Effects of other SNPs should be excluded, for example by using transfected HEFA cells with \textit{TLR9} mutants.

\textit{NOD2} is associated with susceptibility to bacterial meningitis

Although \textit{NOD2} SNPs have been extensively studied and are associated with inflammatory bowel diseases\textsuperscript{34, 35}, we are the first that found an association between \textit{NOD2} SNPs with susceptibility to BM\textsuperscript{36}. \textit{NOD2} mutants were associated with decreased activation of NF\kappa B\textsuperscript{36}, which may be an explanation for an increased susceptibility to BM. Although we used a large cohort of controls, it should be mentioned that \textit{NOD2} SNP mutant alleles are very rare in the Caucasian population. To further investigate this association and to increase statistical power, these analyses should be repeated in sufficiently large patient cohorts. Murine studies showed the importance of \textit{NOD2} in MM. \textit{NOD2} is expressed by murine microglia and astrocytes and becomes upregulated after exposure to \textit{N. meningitidis}\textsuperscript{34}.\textsuperscript{35}
In vitro inflammatory responses of murine astrocytes and microglia are significantly reduced in the absence of NOD2 after stimulation with *N. meningitidis*. Astrogliosis, demyelination, behavioral changes in mice, and increased inflammatory cytokine levels within the CNS in meningococcal infection are reduced in NOD2 knockout mice. These studies indicate that NOD2 represents an important component in the generation of CNS-damaging inflammation following meningococcal infection and SNPs in NOD2 potentially affect the degree of inflammation.

**Genetic variation in toll-like receptors are associated with post-meningitis hearing loss**

BM is the most common cause of acquired hearing loss in children and an important cause of deafness in adulthood. Moreover, 7% to 36% of patients develop permanent sensorineural hearing loss. The resulting social and educational impairments can be devastating to the individual and to society. By identifying children (genetically) at risk for development of post-meningitis hearing loss, early rehabilitation may lessen long-term adverse outcomes.

During BM, infection spreads most likely from the subarachnoid space through the cochlear aqueduct to the perilymphatic space, causing a suppurative labyrinthitis. The inner ear, similar to the brain, is an ‘immunopriviliged’ site in which bacteria can replicate easily since number of immunocompetent cells is small and opsonizing agents are virtually absent under normal conditions. However, bacterial components are recognized by pathogen recognizing receptors (PRRs) initiating the immune response and cytokine transcription by resident immunocompetent cells. A massive immune response leads to collateral disruption of the blood-labyrinth barrier, hair cells and apoptosis of neurons in the spiral ganglion, leading to labyrinthitis ossificans.

We found an association of TLR4 SNPs (combined with TLR2 or TLR9) with post-meningitis hearing loss. There is increasing evidence for a role of TLRs in mediating cochlear damage in meningitis. Pneumolysin, recognized by TLR4, has been identified as a mediator of cochlear damage by its direct cytotoxic effects and via activation of the immune response. In guinea pigs, meningitis due to pneumococci that are deficient in pneumolysin (a TLR2 ligand), causes less hearing loss than meningitis due to wildtype pneumococci. The innate immune response of TLR2/TLR4 double-knockout mice to intracisternal pneumococcal infection was more severely impaired than that of mice lacking only TLR2 or TLR4. Activation of these receptors in the cochlea activates myeloid differentiation primary response gene-88 (MyD88), which in turn activates NFκB. MyD88 knockout mice with PM showed less cochlear inflammation than infected WT mice. Hyporesponsiveness to LPS may result in higher bacterial loads in the cochlea of TLR4 mutant carriers and increased MyD88-dependant inflammation and subsequent cochlear damage. Combined
carriership with *TLR9* mutants (conveying a pro-inflammatory SNP) increases the risk to develop hearing loss, while *TLR2* SNPs seem to compensate for the adverse effect of *TLR4* SNPs. All together, these findings indicate that an adequate pathogen recognition system via MyD88 is important for bacterial clearance from both the CNS and the cochlea but is also responsible for inflammatory damage and hearing loss.

Only one other study is known on SNPs in hearing loss after MM. Patients who developed persistent auditory deficit had significantly higher CSF bacterial loads than those without such deficits. The *IL1RN* SNP (formerly associated with enhanced IL-1β production in vitro^45^) was associated with protection against auditory deficits^31^ but also with increased risk of death in patients with meningococcal disease^46^.

A pro-inflammatory state may protect against HL via reduction in the CSF bacterial load, but not against cytokine dysregulation seen in fulminant meningococcal sepsis.

**Prediction of post-meningitis hearing loss and academic and behavioral limitations**

Many prediction rules are developed, while only a fraction of these studies are used in clinical practice. This is mainly due to lack of external validation and reduced accuracy when a prediction rule is validated in new patients^47^.

We performed external validation on two pre-existing prediction rules for post-meningitis hearing loss and academic and behavioral limitations.

Sensorineural hearing loss is not always noticeable or present directly after treatment of BM, leaving up to a quarter of cases not or never diagnosed^39^.

Especially in young children, hearing loss may only be discovered when formal assessment is done. Even mild impairment in hearing abilities may dramatically impair auditory, linguistic, communication and learning skills. The only current treatment option in complete and profound hearing loss is cochlear implantation which may only be possible in a critical period^38^.

A good opportunity for hearing restoration could disappear within weeks^48^ since labyrinthitis ossificans makes implantation extremely difficult or even impossible. For these reasons a prediction model as developed by Koomen *et al.* identifying children at risk for hearing loss after meningis has been developed. The scores and the matching probability of hearing loss were visually presented in a nomogram for use in clinical practice (figure 2). Although we used a small cohort, we were able to successfully validate this model in an independent cohort of Dutch BM survivors.

Despite the recommendation to perform auditory evaluation in all meningitis patients^38^, this prediction rule may nevertheless be valuable because of the following reasons. First, it may be helpful to determine the frequency of auditory testing and duration of follow-
up, given the highly unpredictable course of post-meningitis hearing loss. Fluctuation and progression occur and dramatically influence the opportunities for cochlear implantation. Most cases occur within the first year after infection, but cases of post-meningitis hearing loss have been described after several years. Second, a prediction rule can create awareness of the importance of hearing evaluation in clinicians and the parents in an early stage of disease. The prediction rule must be easy to access, for instance on a special website where clinicians can easily calculate the risk for hearing loss. Third, BM and subsequent morbidity is considerably more prevalent in developing countries where adequate follow-up, hearing tests, financial resources and support in case of auditory deficits are scarcely available. Prediction models may help to perform hearing evaluation.

**FIGURE 2.** This nomogram can be used to determine the predicted probability of hearing loss given the set of predictor variables. The number of points per predictor variable can be read from the top line. By adding these together, an estimate of the probability of hearing loss can be obtained using the “Total Points” line. For example, a child with a duration of symptoms >2 days (10 points), a CSF glucose level ≤0.6 mmol/l (15 points), and ataxia (36 points) has a score of 61, corresponding to an estimated probability of hearing loss of approximately 0.3.

Adapted from Koomen I et al. Pediatrics 2003;112:1049-1053
in at least high-risk groups. Another research group has developed and validated a prognostic model to predict the risk for unfavorable outcome of BM in the Netherlands, but this model could not be used in developing countries. New prognostic models have to be developed for developing countries in a sufficiently large series of unselected patients.

Similar to a prediction rule for post-meningitis hearing loss, a prediction rule for behavioral and academic limitations in survivors of BM has been developed based on nine clinical predictors. Neuropsychological, behavioral and academic problems have been described to occur in over 20% of childhood BM survivors. External validation in an independent Dutch cohort of school-age children with a history of BM was not successful. Discrimination of the validation cohort was poor (an area under the curve of 0.53), which means there is much overlap in risk scores between patients with- and without academic or behavioral limitations. Validation failure may be due to several factors. Selection bias may have occurred. Gender was significantly different between the development and validation cohort which may lead to an overestimation of the risk in the development cohort. Although expert neuropsychological testing was performed in all children, the outcome “academic and behavioral limitations” is a highly subjective outcome variable determined by several different complex factors, while for a successful validation, simplicity is essential. Prevalence of the outcome was similar in both cohorts. Furthermore, information bias may contribute to differences in patient characteristics. Although using the same methodology, the validation cohort was collected 10-15 years later by different researchers. However, there was no significant difference in distribution of the predictors of the prediction rule, except for gender distribution between both cohorts. In summary, prediction of behavioral and academic limitations in clinical practice is not possible at this moment. Recommendation of careful follow-up of all post-meningitis children may enhance early detection and treatment of these limitations. A valid prediction model should be developed in a new cohort with prospective testing of learning and behavioral performance.

**Prediction of post-meningitis hearing loss including high-risk genes**

Although in univariate analysis TLR SNPs were significantly associated with hearing loss, addition of these high risk genes did not result in a significant improvement of a clinical prediction model for hearing loss after BM. Results are consistent with findings of other studies. Clinical factors, in contrast to genetic factors are intensively used in prediction in clinical practice in other inflammatory- and especially non-inflammatory diseases. Almost without exception, the genetic risk models (including 3-40 SNPs) had lower AUC values than the clinical models. However, most studies focused on diabetes, while only few studies focused on infectious diseases. Our study is the first to combine genetic and clinical data in a prediction rule for meningitis. Thus, there are many opportunities for
improvement; more studies including more combinations of genes and improvement in methodology have to be performed.

Other genetic association studies
For a complete understanding of the innate immunity and phenotype of the disease, it is important to consider genetic variation in not only pathogen recognition receptors, but all following steps following pathogen recognition as well, including cell surface proteins, complement factors, co-receptors, more signal transduction peptides involved in pathways of TLRs and NODs, cytokines and chemokines.

Potential relevant SNPs in BM are extracted from other studies with apparently conflicting results, probably due to methodological shortcomings. Studies focusing on SNPs in meningitis particularly were scarce at the start of this thesis but numbers of papers on this subject are rapidly increasing in the very recent years. Significant associations with susceptibility to BM were found for MBL and macrophage migration inhibitory factor (an upstream pro-inflammatory cytokine). Three Dutch studies revealed that a common variant in C3 was associated with protection against BM and C5 was associated with poor outcome of PM. CARD8 and NLRP1 are associated with poor disease outcome in 531 BM patients. Levels of IL-1β and IL-18 were elevated in the CSF of patients compared to controls and were associated with development of systemic complications and poor prognosis, but were not associated with these CARD8 and NLRP1 SNPs. It seems that variation in cytokine levels affects outcome of BM and may be determined by several different (unknown) SNPs. Measurement of levels of specific cytokine levels in carriers of the aforementioned high risk genes provide a good opportunity to further explore its clinical consequences.

Next to innate immunity genes, SNPs in other mechanism involved in pathogenesis of BM may be of interest. For instance, SNPs in single and combined base excision repair genes (involved in the repair of oxidative DNA damage in the brain) were associated with BM and associated with altered IgG productions and reduction in levels of IL-6, IL-1RA, chemokine ligand-2 and IL8. SNPs in the β2-adrenoceptor, which is used by N. meningitidis and S. pneumoniae to cross the endothelial cells of the BBB, were formerly associated with altered downregulation of receptor expression and in this study with increased susceptibility to BM.

As described before, several studies have been published on polymorphisms in genes controlling the host response to meningococci and pneumococci. Two meta-analyses revealed that polymorphisms or haplotypes in IL1RA, surfactprotein A2, carcinogenic embryonic antigen cell adhesion molecules (CEACAM)3, CEACAM6 and complement factor H were associated with susceptibility and SERPINE1 and interleukin (IL)1β and IL1RN
were associated with mortality in meningococcal disease. The authors concluded that polymorphisms in TLRs did not increase susceptibility to meningococcal disease, but did not focus on meningitis exclusively as in our studies. SNPs in mannose binding lectin (MBL), NFkBIA, NFkBIE, protein tyrosine phosphatase (PTPN22) and Toll/interleukin-1 receptor domain containing adaptor protein (TIRAP) were associated with susceptibility to pneumococcal disease. TIRAP/Mal acts as a bridging adaptor recruiting MyD88 to TLR2 and TLR4. Patients simultaneously carrying polymorphisms in TLR4 and TIRAP had a significantly higher risk of severe Gram-negative infections after surgery and reduced cytokine production in pneumonia patients and ex-vivo in monocytes stimulation assays. Since we found significant results for TLR2 and 4 combined, future studies on BM should include TIRAP genes as well.

Strengths
The combination of basic immunogenetic research with clinical data and new statistical methods with potential translation to clinical practice makes this thesis of interest for a wide spectrum of different fields of research. We focused exclusively on meningitis patients to find the host genetic determinants of this acute and severe infection. We recruited the largest cohort of childhood MM survivors that have been published to our knowledge and found very significant results. This cohort is unique in focusing on meningitis-patients only with microbiological confirmation of all cases. The advantage of inclusion of only survivors of BM allowed us to study genetic variation in the long-term outcome of the disease, for example hearing loss. All cases as well as controls were Dutch Caucasians from different regions in the Netherlands, which makes both groups genetically and geographical comparable.

Our findings emphasize the importance of pathogen recognition receptors in BM, and replenish results of other high quality studies with new genetic information. Increasing convincing evidence for our hypothesis on functional consequences of the TLR9-1237 SNP was published independent from our findings. We combined multiple high-risk genes in the same pathways, a concept that is used in only few other studies.

Focusing on the details of the immune response during BM may not only lead to new hypotheses and insights in the pathophysiology of meningococcal- and pneumococcal infections, but other inflammatory conditions as well. This may ultimately lead to development of new adjunctive therapies, resulting in decreased mortality and morbidity. In order to achieve this, still many steps have to be taken. At this moment the major contribution of genetic association studies is to provide a better understanding of pathophysiologic processes.
Critical notes
Although we have found some very significant results, a real effect of these SNPs is not indisputably proven. SNP association studies are hypothesis driven, but do not prove causation. Additional functional studies are needed to explain the biological mechanisms of a functional SNP. The numbers of patients are relatively small for a study of polymorphisms. Our data should be validated in an independent Dutch Caucasian cohort as well as other ethnic patient groups. We did combine two different cohorts that were collected in different periods, and found no significant differences in SNP distributions between both cohorts. The second cohort consisted of only 74 children and should be extended.

Survivors of BM were retrospectively included in our study. This is inevitable with a disease that happens acute and is rare, but it might induce selection bias, recall bias and missing data. This was the case in our study; percentages of missing data ranged from 0 to 6% in SNPs and 0 to 40% in clinical variables. This is partly due to usage of buccal swabs for genetic analysis. This is a widely accepted method to collect DNA which leads to a higher participation rate since patients can take self-samples at home, but the drawback is potential low quality of DNA.

Our cohort of PM survivors was considerably smaller (n=88) than MM patients and we could nearly find significant results for this group. This cohort should be extended with PM patients. However, these PM patients contribute to the total BM group and have many similarities with the pathogenesis of MM. DNA from children with fatal BM (PM in particular) who suffered the most severe infections was not available. We expect however, that including DNA of fulminant cases will only strengthen the associations we found. Statistics were performed for 11 SNPs and up to 13 clinical variables which may lead to false-positive results as a result of multiple testing. However, associations remained significant after correction for multiple testing.

Future perspectives
Darton et al. first showed the direction that genetic research in meningitis should be going. They included both host and pathogen factors in a stratified analysis and correlated for host factors, bacterial DNA load and bacterial serogroup with clinical outcome of meningococcal disease. As an increasing number of bacterial genomes is becoming available, assessing the role of microbial genes in virulence and pathogenesis should be included in order to get a complete understanding of these interactions and prognostication of meningitis. Piet et al. reported that polymorphisms in the factor H binding protein of the meningococcus was associated with septic shock and adverse outcome in meningococcal meningitis. In another French study of 962 cases of children with MM it was found that two specific
phenotypes/genotypes within serogroup B and C meningococci were associated with higher mortality than other phenotypes. Another pathogen protein of interest may be PorB, a component of the meningococcus that is recognized only by TLR2 which has been shown to be variable between different pathogenic and non-pathogenic types of meningococci. Studying both PorB variation and TLR2 genetic variation in susceptibility to MM will allow us to fully understand this interaction. Combining host genetic variation and specific serogroups or serotypes of meningococci represent an interesting focus of further research but first extension of our cohorts is needed to increase the power.

In addition to candidate gene studies, next generation sequencing and genome-wide association studies (GWAS), in which several hundred thousand genes and more than a million SNPs are assayed in thousands of individuals, represent a powerful new tool for investigating the genetic architecture of complex diseases. Recently, the first GWAS performed in 475 cases of meningococcal disease identified one susceptibility locus in the complement factor H region. These massive GWAS studies do not make the candidate gene approaches redundant. Finding the tagging SNP only narrows the search region on the gene. It is still necessary to identify the candidate gene. A problem with GWAS studies is the missing heritability, because variants of low minor allele frequency, defined as roughly 0.5% are not sufficiently frequent to be captured by current GWAS genotyping arrays. However, these variants are captured in candidate gene studies, especially in extremes of a quantitative trait such as BM.

Most genetic association studies focus on the clinical outcome of a disease. Focusing on the more direct link, as in endophenotypes allows us to study the biological consequences of a SNP and moreover, eliminates confounding factors. This includes studying SNPs for differences in the mRNA expression level, protein levels in circulation or whole pathways. A good illustration how to combine SNP data with patient and mice-experimental data and translation to potential treatment, is a study of Woehrl et al.. Investigating the role of common genetic variants in the complement system in outcome of BM, they found that the C5 (rs17611) SNP is associated with unfavorable disease outcome and lower CSF leukocyte counts. This variant was associated with reduced serum C5. Further, C5 levels in CSF of patients correlated with Glasgow coma scale, death and unfavorable outcome. C5a receptor-deficient mice displayed decreased morbidity compared with WT mice. Adjuvant treatment with C5-specific monoclonal antibodies prevented death in all mice with PM. This may provide interesting opportunities for human therapies.

In this thesis the focus is especially on pathogen recognition system. In order to achieve a complete genetic risk profile, additional studies should combine these high risk genes with more different signal transduction peptides, cytokines and complement genes involved in specific pathways that are important in the pathogenesis of meningococcal
or pneumococcal infections. In order to facilitate the comparison and fusion of multiple genetic association studies all studies should meet the same criteria to improve the quality of these studies. Important quality criteria for immunogenetic association studies are listed in table 1.

**TABLE 1. Recommendations for genetic association studies in infectious diseases**

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<th>Diagnosis</th>
<th>Microbiological confirmation of the diagnosis</th>
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<td>A clear definition of the diagnosis</td>
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<td>Methods</td>
<td>Study design (preferred prospective)</td>
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<td>Sample size and power analysis</td>
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<td>Correction for confounders</td>
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<td>Blinding of laboratory personnel</td>
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<td>Correction for multiple testing</td>
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<td>Replication of positive findings (external validation)</td>
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<td>Replication in other ethnic groups</td>
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<td>Cases</td>
<td>Clear definition of patient characteristics</td>
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<td>Controls</td>
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<td>Concurrence with the Hardy Weinberg equilibrium</td>
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<td>Equivalent exposure to nasopharyngeal carriage</td>
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Furthermore, it is important to take into consideration the non-significant SNPs as well. Current knowledge on genetic association studies may be biased by publication bias, which means that positive results are more likely to be published. Since big cohorts in different ethnicity groups are needed to study SNPs, institutions should cooperate instead of being competitive in order to collect as many cases and controls as possible and increase statistical power. Especially in case of rarely occurring diseases like meningitis, research groups should work together in order to increase statistical power.

**Potential implications in therapy of BM**

Potential therapies need to be directed towards both pathogen eradication with antibiotics as well preventing the injurious effects of the ensuing immune response. The latter is currently achieved with steroid treatment; however, this is a rather non-specific approach and the targeting of specific harmful pathways cannot be realized. Owing to dysregulated inflammatory cascades that have been implicated in the pathophysiology
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of BM, interference with TLR activity may represent a strategy for damping meningeal inflammation and improving disease outcome. Mouse models clearly demonstrated that disruptions in MyD88 or TLR2/TLR4 and TLR9 were detrimental to the host; therefore risk of uncontrolled infection as a consequence of impaired bacterial eradication during TLR antagonist therapy may surpass any potential benefit. Thus any potential antagonist therapies must be appropriately scrutinized. Genetic variation studies may help to distinguish between detrimental and advantageous effects of specific TLR activation during BM.

In the near future the most important benefit will come from vaccination, which can prevent outbreak of infection in endemic areas. Genetic association data may be used in vaccination strategies. For example, pre-clinical data support the use of TLR9 agonists as vaccine adjuvants. Re-interpreting CpG therapy trial results, taking into consideration the genotype of the participants, may therefore be of interest, since the global results may have been masked by the presence of the relatively common TLR9-1237 SNP. Ultimately, the therapeutic applications of CpG should be individually tailored as they may prove to be either detrimental or more effective for individuals harboring the TC genotype.

The next step is to enable advances in biomedicine and genomics to be responsibly translated into effective ways to prevent illness and provide healthcare. These processes are fueled by the field of Public Health Genomics (http://www.phgfoundation.org), which aims to bridge the gap between research and clinical practice studies into beneficial interventions and ensure they will be effectively implemented, for which multidisciplinary efforts are needed (25, 26). For example, a current project includes the implications of whole genome sequencing technologies for health in the UK. Furthermore, The Human Genome Project offers new opportunities for the promotion of population health. Benefits are anticipated through more effective personalized preventive care, disease treatments with better specificity and innovative drug therapies. In general, the success rate of timely translation of genome-based technologies to commercially feasible products or services with applicability in health care systems is significantly low. Both industry and scientists neglect health policy aspects when commercializing their technology. Lal et al. developed a new model of valorization to optimize integration of genome-based technologies into the healthcare system through early involvement of stakeholders and networking. Currently, applications of the results to individual patients is still rare and currently restricted to rare diseases or in pharmacogenomics (dose adaptations of drugs e.g. Warfarin). In summary, despite the ongoing progress in genetic association studies in complex diseases, there are many barriers to cross before SNPs will be used in clinical practice.
Final conclusion

Although CNS inflammation is necessary to guarantee sterility of the CNS, its injurious properties are also evident. An adequate but balanced inflammatory response inside the CNS is essential in limiting adverse outcome of disease. TLRs and NOD2 polymorphisms may play an important role in the balance between beneficiary and injurious effects of inflammation in the CNS. These high-risk genes may in the future be used for identifying patients at risk for meningitis and sequelae.
REFERENCES


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