Author's response to reviews

Title: Toll-like receptor 9 polymorphisms are associated with severity variables in a cohort of meningococcal meningitis survivors

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Author's response to reviews: see over
Reviewer's report (1)

Title: Toll-like receptor 9 polymorphisms are associated with severity variables in a cohort of meningococcal meningitis survivors

Version: 4 Date: 5 December 2011

Reviewer: Marieke Emonts

Reviewer's report:

Review of

Toll-like receptor 9 polymorphisms are associated with severity variables in a cohort of meningococcal meningitis survivors

MS Sanders et al.

Major Comments

1. In the abstract the authors state that they relate TLR9 polymorphisms to predictors of death and severity in survivors. Studying death predictors in a cohort of survivors will give a skewed picture. In the methods section it is however only stated that these are severity markers, while nothing is stated about mortality.
   - We agree on the fact that the sentence about “death” in the abstract is misleading since our study focuses on survivors. We changed this sentence into “adverse outcome.” In our literature search for clinical variables associated with adverse outcome, we did include variables associated with death because we consider this as a severe course of disease. We hypothesize that inclusion of children who died in our analysis will only make our associations stronger.

2. Why are continuous variables dichotomized? This will result in a loss of statistical power. In the results section, the continuous outcome variables are presented as both continuous and dichotomised. Advise to limit this to continuous variables. (this also limits the number of tests, added to the already 13 for both SNPs). Why use two dichotomous variables for CSF leukocyte count limits 600 and 1000)?
   - Variables were dichotomized based on cut-off points that have been associated with severity of meningitis in literature. Cut-off points of leukocytes of less than 600 (des Portes et al.) and 1000 (Weisfelt et al.) – extra references are added in method to clarify – were in other studies associated with poor outcome. We choose these cut-off values to distinguish between severe and less severe, because based on continuous variables only this is not always clear.

3. A p value of <0.05 is considered significant. However, 13 outcome variables were tested. A correction for multiple testing is in place, which would result in no remaining significant results.
   - We have performed corrections for multiple testing and added a discussion to the Discussion section of the manuscript. Although after correction for multiple testing not all findings remain significant, trends are observed, moreover, all findings we found are in line with our hypothesis and current literature on TLR9 functional consequences, while other 10 variables did not reach a trend or significance.
4. Genotype distributions were checked for deviations of HWE. In a previous study the TLR9 +2848 polymorphism was observed to be associated with susceptibility to MM in this cohort. This implies automatically a deviation. Was this checked in a control population?
- Yes, the controls as described in the former study (Sanders et al., CID, 2011) were in HWE for the TLR9+2848 SNP as well as the TLR9-1237 SNP.

5. TT is a wild type genotype, not allele (results page 10). Please check use of the terms ‘carrier’, ‘allele’ and ‘genotype’ throughout the text.
- We agree on this point and have adjusted this in our article.

6. Table 2. As bacterial meningitis is associated with decrease CSF/blood glucose ratio, it is surprising that the association observed for the TLR9 polymorphism is associated with decreased numbers of positive blood cultures as well as a decreased CSF/blood glucose ratio. Please explain.
- Our explanation for this remarkable finding is that decreased CSF/blood glucose ratios reflect a higher consumption of glucose by immune active cells. Increased immune activation in the circulation may lead to a decreased number of positive blood cultures. Ostergaard et al. studied 153 patients with pneumococcal meningitis, no significant difference in CSF WBC was observed between patients with or without bacteraemia at admission but there was a significant correlation between CSF and blood WBC. We suspect that the genetically determined immunological response to a pathogen in the circulation or in the CNS (and the intensity of inflammation) contributes to the number of leukocytes in the CSF as well as in the circulation and secondary bacteraemia and CSF/blood glucose ratio.

7. What is the effect of not only increased (in silico) binding of TLR9 -1237 C variant to NFkB, but also to RelA, and STAT3? In other words: what do you expect when not NFkB binds, but preferably RelA or STAT3?
- The RelA gene, encoding for transcription factor p65, binds to NFkB to form the NFkB complex. STAT3 is able to activate transcription in the nucleus in response to cytokines. We added this to figure 1.

8. The authors state that (page 14) TLR9 polymorphisms have a small but important contribution to warrant the balance between beneficiary and injurious effects of inflammation in the CNS. This was not shown from the results. I would then have expected an association with neurological outcome, which was not demonstrated.
- This sentence was changed into TLR9 polymorphisms have a small but possibly important contribution to warrant balance between beneficiary and injurious effects of inflammation in the CNS. Our study shows that that may have functional consequences in the acute course of diseases, whether this contributes to important long-term sequelae needs further exploration. Other studies focussing on the role of SNPs in the long-term consequences of BM are needed.

9. Conclusions: The remark that these SNPs result in a lower chance of developing meningitis when colonized with N. meningitidis should be limited to TLR9 +2824 SNP. 9 page 16).
- We agree on this comment, and have adjusted accordingly.

Minor comments

1. Where all individuals CSF cultures positive for N. Meningitidis?
-Yes, the diagnosis meningitis was based on the demonstration of bacteria or bacterial antigens in the CSF as collected by the Netherlands Reference Laboratory for Bacterial Meningitis.

2. CRP > 100 is considered to be related to more severe disease. This is in contrast to meningococcal sepsis in which a low CRP at presentation is associated with more severe disease and mortality.

- Le Clerc et al and Hazelzet et al. associated CRP <100 with higher mortality in neonatal sepsis. On one other (too) small Turkish study (n=42) on purulent meningitis, high CRP at admission, and a low CRP >3 days was associated with mortality and sequelae. In our opinion patients with meningococcal meningitis are not completely comparable with septic shock patients, since different pathophysiologic pathways are activated. Despite the fact that meningitis is often accompanied with sepsis, this is not always the case. Hypothetically, in the CNS a decreased immune response may be advantageous by protection of healthy neurons to immune mediated cerebral oedema, release of cytotoxic factors and cytokines. We can not answer this question with this study, but this provides an interesting subject for further research.

3. Surprisingly CRP is missing for many of the individuals while it is usually a parameter that is checked in ill children. Can you please explain?

- Our study population is included retrospectively (time period of 1990 to 2001), and most of the children were born before 1995. In that period CRP was a new parameter that was in that time not yet commonly determined.

4. Table 3: please describe TLR9 haplotypes, reference only is not sufficient.

- TLR9 haplotypes are added in the upper row of table 3, we agree this explains the table better.

5. Discussion page 13 and 14: The authors suggest a protective effect of TLR9 polymorphism, preventing bacteremia and increase leukocyte influx in the CNS. Did the individuals with positive bloodculture but no clinical signs of sepsis have increased CNS leukocyte influx compared to the patients with both positive bloodculture and clinical signs of sepsis?

- Yes, we conclude that in all patients with a positive blood culture, the mean CSF leukocyte count is significantly higher in patients without sepsis, compared to patients with sepsis (see table). Overall, a significantly lower number of CSF leukocytes is seen in patients with a positive blood culture (2813 leukocytes/ µL) compared to means of 5700 leukocytes/ µL in sterile blood cultures. Blood leukocyte counts are also significantly decreased in bacteraemia patients compared to patients without bacteraemia (15.590 x 10^9/L vs 18.724 x 10^9/L). It is tempting to speculate that that TLR9 SNPs by an enhanced immune response protect against bacteraemia and systemic inflammation, and may result in an increased efflux of leukocytes into the CNS.

<table>
<thead>
<tr>
<th>Blood culture</th>
<th>Sepsis</th>
<th>No sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. meningitides detected</td>
<td>1893/µL</td>
<td>4194/ µL</td>
</tr>
</tbody>
</table>

Minor issues not for publication
1. Both in the abstract and methods section (page 8) CSF/blood glucose ratio instead of CSF blood/glucose ratio.
   - Typo is corrected.

2. How was controlled for pre-existing hearing loss?
   - Cases of conductive hearing loss were not included and patients with ‘complex onset’ of meningitis (defined as meningitis secondary to immune deficiency states, cranial trauma, CNS surgery, and CSF shunt infections), cognitive or behavioral problems prior to meningitis and relapsing meningitis were excluded. Hearing status has not been tested prior to meningitis, but by exclusion of aforementioned variables, we reduce the chance of pre-existing hearing loss.

3. Reference numbers are listed from 1-21 and then 0-3, please adjust.
   - References are up to date in this version. Two very recent references on functionality of the TLR9-1237 SNP have been added to the paper (Sam Adogu 2010, and Carvalho 2011).

4. Table 1 CSF leukocytes (/μL or μL⁻¹)
   - This was adjusted in table 1.

5. Rephrase sentence page 13: TLR9 -1237 was….TLR9 -1237 C variant (figure 4. Now it says: TLR9 -1237 was associated with significantly increased binding of NFκB, RelA, and STAT3 to the TLR9 -1237 C variant (figure 4). Do you mean: The TLR9 -1237 C variant was associated with significantly increased binding of NFκB, RelA, and STAT3 (figure 4). ?
   - This detail was corrected in the text.

6. Suggest to omit statement on Public health genomics, bottom page 14. It does not add to the content of the manuscript.
   - Though this statement is a side step of our study, it explains what the ultimate goal of these types of studies is and what is needed in order to benefit from these studies. We shortened this statement in to one short sentence.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, and I have assessed the statistics in my report.

Declaration of competing interests:

I have worked with some of the authors on this paper. This was however in no way related to the work presented here, nor does it influence my review on this paper.
Reviewer's report (2)

Title: Toll-like receptor 9 polymorphisms are associated with severity variables in a cohort of meningococcal meningitis survivors

Version: 4 Date: 4 January 2012

Reviewer: Uwe Koedel

Reviewer's report:

The authors investigated the genotype frequencies of two TLR9 single nucleotide polymorphisms (SNPs) and their association with 13 clinical variables in 390 children who survived meningococcal meningitis (MM). The TLR9 -1237 TC and CC allele were found to be associated with a decreased incidence of a positive blood culture as well as an increased cerebrospinal fluid (CSF) pleocytosis. The same holds true for the TLR9 +2848 AA mutant allele. All the other variables (e.g., hearing loss, ICU admission, vigilance level, and presence of convulsions at admission) did not differ between children carrying different alleles. These data suggest an important (albeit minor) role of TLR9 SNPs in the host defense against meningococcal infection of the CSF and are in line with recent findings from this group and others that showed [i] a reduced susceptibility to MM in children carrying the TLR9 +2848 A allele (Sanders et al., Clin Infect Dis 2011:52, 475-80) and [ii] an involvement of TLR9 in immunological recognition of Neisseria meningitidis in vitro and in vivo (Mogensen TH et al., J Leuko Biol 2006: 80, 267-77 and Sjolinder H et al., Infect Immun. 2008: 76:5421-8).

In general, the manuscript is well written and clearly structured. The data are interesting and provide further evidence for a role of TLR9 in the immune response to meningococcal infection in humans. A few alterations would improve the manuscript.

Major Compulsory Revisions:

[1] Recent experimental work has provided substantial evidence that [i] in meningitis, pathogens migrate from the blood to the CSF and vice versa, and [ii] high bacterial concentrations are key determinants for the development of both meningitis and secondary bacteremia. Accordingly, a protective effect of the TLR9 SNPs against primary meningococcemia - a prerequisite for the meningeal invasion, as stated by the authors – should result in a reduced frequency of meningitis (as previously shown by the authors) and/or a mild course of meningitis. The latter could be expected since the degree of CSF pleocytosis depends among others on the infectious dose. In my opinion, it seems to be more likely that the decreased incidence of positive blood cultures in children carrying TLR9 -1237 C and TLR9 +2848 AA alleles may represent a reduction in the occurrence of secondary bacteremia due to more pronounced (and efficient) host immune response in the CSF. The authors should include a discussion of this topic in their manuscript.

- We agree on this interesting vision and have included this topic in our manuscript.

Moreover, the authors could test for associations between the presence of positive blood cultures and both, the duration of clinical illness and the start of antibiotic therapy before admission.
Duration of clinical illness was not significantly associated with a positive blood culture (p = 0.073), though it is an interesting question. Unfortunately, we have no decent information on use and type of antibiotic therapy before admission.

[2] The author did not detect any association between the TLR9 SNPs and important clinical severity variables including postmeningitic hearing loss, presence of convulsions at admission, and ICU admission. However, important severity measures are lacking, namely the length of the hospital stay and neuropsychological impairments.
- Both variables were additionally tested. No significant differences in length of hospital stay were observed. We did not include the variable “neuropsychological impairments, in this study, because we consider this as a very subjective variable, which is too speculative for this study, in which we look for functional explanations. Work on the prediction of neuropsychological impairments after meningitis is in progress.

In previous studies (Koomen I et al., ActaPediatr 2003 and Developmental Medicine & Child Neurology 2004), van Furth’s group investigated the frequency of neuropsychological sequelae in survivors of MM. There is a considerable overlap between the patient cohorts of this and the previous studies. Therefore, I tend to think that it should be doable to include these measures into the actual association analysis.

[3] The authors mention the results of a so-called in silico regulatory SNP detection method in the Discussion section. This method was already used and described in detail in their previous publication in Clin Infect Dis. The authors should either delete the figure or, if able to provide new information, describe the method and results in the respective sections.
- The in silico analysis as described in this study was an in silico analysis of the TLR9 promotor region, contributing to a possible explanation for our findings, which is why it is included in our paper. In the other TLR9 article (CID, 2011), we indeed performed an in silico analysis, but this was an analysis of the genome of the meningococcus and the pneumococcus. Both methods are distinctly different, and therefore we have adapted the text to better reflect the results of this in silico analysis.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
'I declare that I have no competing interests'