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(Cruijff)

8 Single nucleotide polymorphisms in innate immune response genes affect the susceptibility to meningococcal meningitis: genetic traits and pathway analysis

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ABSTRACT

Bacterial meningitis (BM) is a serious central nervous system infection, frequently occurring in childhood often resulting in hearing loss, learning disabilities and encephalopathy. Genetic variation in pathogen recognizing genes affects susceptibility, severity, and outcome of BM. The aim of this study is to describe the effect of single nucleotide polymorphisms (SNPs) in pathogen recognizing genes on susceptibility to develop BM.

Genotype frequencies of eight SNPs, in five immune response genes, in 391 meningococcal post-meningitis (MM) and 82 pneumococcal post-meningitis (PM) children were compared with 1141 ethnically matched healthy controls.

TLR4 +896 GG predisposed to susceptibility to develop MM (p=1.1 x 10e-5, OR=9.4, 95% CI=3.0-29.2). The *NOD2* SNP8 mutant was significantly more frequent in MM patients compared to controls (p=0.0004, OR=12.2, 95% CI=2.6-57.8). Combined carriage of *TLR2* +2477 and *TLR4* +896 mutants was strongly associated with MM (p=4.2 x 10e-5, OR=8.6, 95% CI=2.7-27.3). A second carrier trait of *TLR4* +896 and *NOD2* SNP8 mutants was also strongly associated with susceptibility to develop MM (p=4.2 x 10e-5, OR=10.6, 95% CI=2.9-38.6).

This is the first study associating SNPs and genetic traits of immune response genes with susceptibility to develop MM, enabling the identification of patients at risk.

BACKGROUND

Susceptibility to infections is determined by genetic variation in human populations as can be concluded from genetic epidemiology studies. An important challenge is identifying the responsible genes and translating these findings into biological mechanistic explanations [1, 2]. Bacterial meningitis (BM) is a severe infectious disease of the central nervous system (CNS). It occurs relatively frequent in childhood and often affects hearing and learning abilities [3, 4]. Immune responses to pathogens which cause BM are primarily aimed at eliminating bacteria from the CNS by recognition of microbial ligands and subsequent triggering of production of specific cytokines, but also contribute to collateral damage to healthy neuronal tissue and adverse outcome [5]. Genetic variation in genes encoding for pathogen recognizing receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD) like receptors (NLRs), leads to enhanced or decreased inflammatory responses in several cell types such as macrophages and epithelial cells [6]. Microglia, the resident macrophages inside the CNS, and neuro-epithelial cells also express TLRs and NLRs. So, genetic variation of these receptors might influence susceptibility, severity and outcome of BM. It was recently shown that two *TLR9* single nucleotide polymorphisms (SNPs) were associated with increased serum levels of IFN-γ in children with cerebral malaria [7]. Several other genetic association studies have shown that SNPs in innate immunity genes were associated with susceptibility to meningococcal and pneumococcal disease, including cases of meningitis [8-10]. We previously described that carriage of the *TLR9* +2848 SNP protects against BM [11].

In this study we focused on genetic variation of innate immune response genes in BM caused by *Neisseria meningitidis* and *Streptococcus pneumoniae*, the two most common causing pathogens of BM in the world [3]. We selected eight SNPs in five immune response genes based on BM pathogenesis which usually starts with nasopharyngeal colonization, epithelial disruption by bacterial components, enabling these bacteria to enter the bloodstream where they replicate and cause bacteremia, might cross the blood-brain barrier (BBB) and multiply in the subarachnoid space [8]. The immune response inside the CNS upon bacterial entrance starts with pathogen recognition by microglia and astrocytes and by non-neural structures in direct contact with the cerebrospinal fluid (CSF), such as dendritic cells and macrophages, all expressing PRRs including TLRs and NLRs. PRR activation triggers an intracellular signaling cascade resulting in the transcription of pro-inflammatory cytokines and chemokines, also inside the CNS [6]. Cytokine induced increased permeability of the BBB and chemokine induced influx of inflammatory cells from the bloodstream into the CNS result in enhancement of the local inflammatory response inside the brain. The clinical consequence is brain edema, raised intracranial pressure, infarction and

neuronal injury [12]. The ability of a host to sense CNS invasion by microbes and to respond appropriately to control the local infection is essential for killing these microbes but the inflammatory response also results in the production of several cytotoxic mediators responsible for damage to healthy neuronal cells and thus for adverse disease outcome [12].

TLR2 and TLR4 are PRRs located on the surface of immune cells and recognize cell wall components of both Gram-positive and Gram-negative bacteria. Animal data have shown that deficiency of TLR2 and TLR4 leads to reduced bacterial clearance from the CNS in response to *S. pneumoniae* infection [13]. NOD1 and NOD2 recognize degradation products of petidoglycan (PGN), an essential component of the cell wall of almost every bacterium. Isolated cultures of murine astrocytes and microglia constitutively express robust levels of NOD2 after exposure to both *N. meningitidis* [14] and *S. pneumoniae* [15]. Cysteine-dependent aspartate-directed protease (Caspase) plays an essential role in apoptosis. Activation of intracellular caspase-1 (CASP1) upon pathogen recognition by TLRs and NODs defends the host against infection by secretion of the pro-inflammatory cytokines IL-1β and IL-18 via the IL-1 receptor, and by the induction of apoptosis of infected cells [16]. CASP1 levels are up regulated in the CSF of patients with BM and correlate with clinical outcome, assessed by the Glasgow Coma Scale and *CASP1* -/- mice intracerebrally infected with *S. pneumoniae* show a significantly attenuated increase of IL-1β, lower CSF leukocytes and an improved clinical status [17].

The aim of this study was to identify the effect of genetic variation in the aforementioned five genes on susceptibility to BM by comparing gene frequencies in post-meningitis children with healthy controls. Genetic association studies frequently focus on a single gene or SNP. We also studied the potential synergistic effects of SNPs by defining traits in biological relevant SNP combinations.

METHODS

Patients

Figure 1 shows a flow chart of patient inclusion in this study. The cohort of school age survivors of meningitis is composed of two independent, comparable cohorts, described by Koomen *et al.* and a validation cohort [18, 19]. All patients were selected from data on bacterial CSF isolates of the Netherlands Reference Laboratory for Bacterial Meningitis. Only Dutch-Caucasian survivors of meningococcal meningitis (MM) and pneumococcal meningitis (PM) were asked to participate in this study. The original cohort consists of children born between January 1986 and December 1994 who survived BM between January 1990 and December 1995. The validation cohort

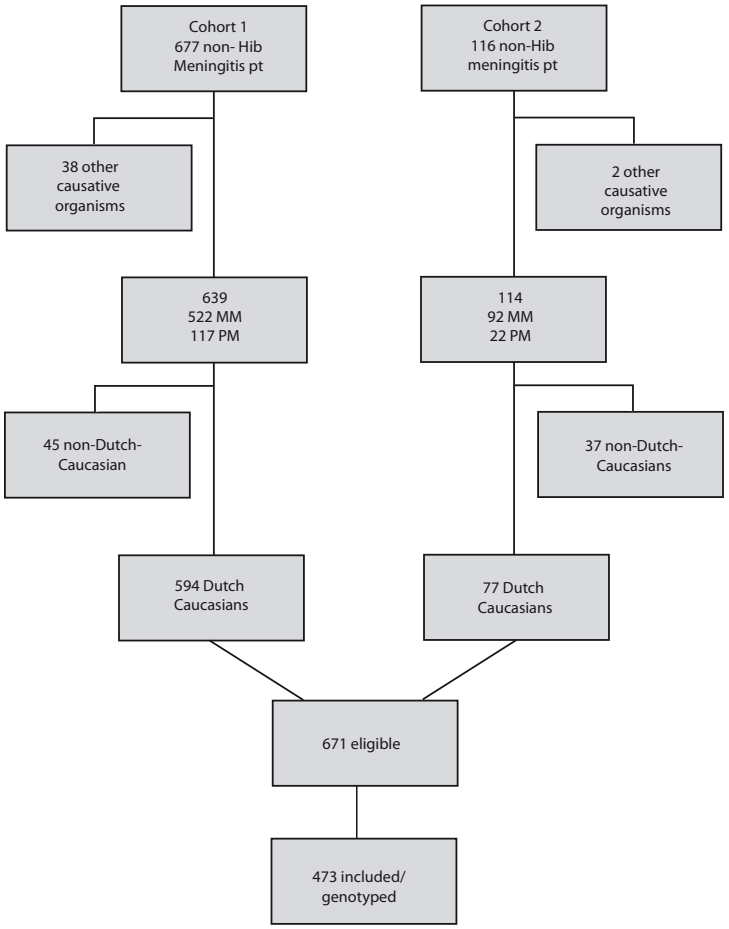

Figure 1. Flow chart of patient inclusion in this study

consists of children born between January 1993 and December 1999 who suffered from BM between January 1997 and December 2001. Clinical characteristics of both cohorts were comparable and no significant differences in genotype distributions were observed between both cohorts.

Six hundred seventy one (671) eligible patients were asked to return a sterile swab after collecting their buccal DNA. Of these patients a total of 473 (70%) gave informed consent and returned a buccal swab. The cohort consisted of 391 former MM patients and 82 former PM patients. The median age of all patients at the time of infection was 2.2 years of age (range 0.1 – 9.5) and 56 % were male. Children with 'complex onset' of meningitis (defined as meningitis secondary to immune deficiency states, cranial trauma, CNS surgery, and CSF shunt infections, meningitis in the neonatal period) or

relapsing meningitis were not included. The Medical Ethical Committee of the VU University Medical Center approved this study.

Controls

Controls were unselected blood donors and population controls, drawn from volunteers at the University Medical Centers of Utrecht and Groningen. Written informed consent was obtained from all subjects, with Ethics Committee approval of both institutions. Genotype data for 1141 control **s**amples were extracted from Immunochip platforms using PLINKv1.07 [20, 21].

DNA isolation

DNA was isolated from the buccal swabs using the following procedure: after addition of 250 µl 10 mM Tris-HCl (pH 7.4) the sample was heated at 96 °C for 10 minutes. After mixing for 10 seconds the swabs were removed and the sample was centrifuged few seconds (14000 rpm). In controls, venous blood (5-10 ml) was drawn and genomic DNA was isolated using standard protocols and 5-100 ng of genomic DNA was used each cycle of genotyping.

Genotyping

All samples were genotyped for the *TLR2* +2477 G>A (rs5743708), *TLR4* +896 A>G (rs4986790), CARD4/*NOD1* +32656 C>A (rs6958571),*CARD15/NOD2* +2209 A>T (rs2066844), *CARD15/NOD2* +2722 G>T (rs2066845) *CARD15/NOD2* +3020 ins C (rs2066847) and *CASP1*-8404 A>G (rs2282659) SNPs by real-time polymerase chain reactions (RT-PCR) using the TaqMan AbiPrism® 7000 Sequence Detection System (Applied Biosystems, UK) and the the LightCycler® 480 System (Roche Applied Science, US),using standard manufacturer's protocols. Twice two independent researchers checked results.

Statistics

Genotypes were compared between cases and controls for MM and PM separately and for all cases of BM together using Graphpad Instat. Hardy-Weinberg tests were used to check the observed genotype distributions in the control population. Fisher's exact tests were used to calculate statistical significance of differences in genotype frequencies between cases and controls. *P*-values < 0.05 were considered statistically significant in single gene analyses. Subsequently, the single genotypes were used to define carrier traits. After correction for multiple testing according to Holm-Bonferroni, *p*-values < 0.0006 turned out to be statistically significant in the trait analyses.

RESULTS

Hardy-Weinberg tests

Genotype distributions of all SNPs in the controls were in Hardy-Weinberg Equilibrium (HWE).

Single gene analysis

Genotype frequencies of BM patients were compared to those in controls and MM and PM patients were also separately compared to controls in order to discover associations between SNPs and susceptibility to a specific pathogen. The results are summarized in *Table 1*. Differences in numbers of cases and controls were due to differences in quality of DNA in the samples. SNPs that could not be genotyped after three PCR test were excluded.

Carriage of homozygous mutant alleles for *TLR4* +896 predisposed to susceptibility to develop BM. Significantly more BM patients than controls were affected (p=1.1 x 10e-5, odds ratio (OR) 9.0, 95% confidence interval (CI) 2.9-27.5). This was even stronger for MM patients compared to controls (p=1.2 x 10e-5, OR 9.4, 95% CI 3.0-29.2). For PM patients the difference was not statistical significant.

Differences in genotype frequencies were also found for *NOD2* SNP 8 when comparing carriage of homozygous mutant alleles with heterozygous or homozygous wild types in the total group of BM patients (p=0.001, OR=10.0, 95% CI=2.1-47.4). MM patients also carried more often homozygous mutant alleles of *NOD2* SNP8 than controls (p=0.0004, OR=12.2, 95% CI=2.6-57.8). The difference between PM patients and controls was not statistically significant.

For the other tested SNPs we did not find differences in genotype frequencies comparing patients to controls.

Carrier trait analysis

With carrier trait analyses we investigated combinations of SNPs. We studied the implication of the combined effect of individual SNPs on susceptibility to BM. Based on associated biological pathways and guided by the results of the single gene associations we tested which combinations of two SNPs showed an enhanced statistical association implying synergistic effects of combined gene functions. *Table 2* shows the traits significantly associated with susceptibility to BM.

Combined carriage of homozygous mutant alleles *TLR2* +2477 and *TLR4* +896 strongly enhanced the predisposition to develop BM (p=3.4 x 10e-5, OR=8.4, 95% CI=2.7-25.9). This effect was even stronger for MM patients compared to controls (p=4.2 x 10e-5, OR=8.6, 95% CI=2.7-27.3), however for PM it did not reach statistical significance.

Table 1. Genotype distributions in bacterial meningitis survivors versus controls

SNP	Total BM N (%) 473	MM N (%) 391	PM N (%) 82	Controls N (%) 1141
TLR2 +2477	466	384	82	1141
GG	418 (89.6)	345 (89.8)	73 (89.1)	1041 (91.2)
GA	46 (9.9)	37 (9.6)	9 (10.9)	96 (8.4)
AA	2 (0.5)	2 (0.6)	0 (0.0)	4 (0.4)
P-value*	1	0.6	1	
OR (95% CI)	1.2 (0.2-6.7)	1.5 (0.3-8.1)	1.5 (0.1-29.0)	
TLR4 +896	456	376	80	1141
AA	401 (87.9)	328 (87.2)	73 (91.2)	1001 (87.7)
AG	41 (9.0)	36 (9.6)	5 (6.3)	136 (11.9)
GG	14 (3.1)	12 (3.2)	2 (2.5)	4 (0.4)
P-value*	< 0.0001	< 0.0001	0.05	
OR (95% CI)	9.0 (2.9-27.5)	9.4 (3.0-29.2)	7.3 (1.3-40.4)	
NOD1 +32656	450	372	78	1141
AA	260 (57.8)	210 (56.5)	50 (64.1)	663 (58.1)
AC	161 (35.8)	136 (36.6)	25 (32.1)	414 (36.3)
CC	29 (6.4)	26 (6.9)	3 (3.8)	64 (5.6)
P-value*	0.5	0.3	0.8	
OR (95% CI)	1.2 (0.7-1.8)	1.3 (0.8-2.1)	0.7 (0.2-2.2)	
NOD2 SNP8	463	381	82	1141
CC	414 (89.4)	341 (89.5)	73 (89.0)	1063 (93.2)
CT	41 (8.9)	32 (8.4)	9 (11.0)	76 (6.7)
TT	8 (1.7)	8 (2.1)	0 (0.0)	2 (0.1)
P-value*	0.001	0.0004	0.2	
OR (95% CI)	10.0 (2.1-47.4)	12.2 (2.6-57.8)	1.7 (0.8-3.5)	
NOD2 SNP12	454	379	75	1141
GG	443 (97.6)	369 (97.4)	74 (98.7)	1096 (96.1)
GC	8 (1.8)	8 (2.1)	0 (0.0)	45 (3.9)
CC	3 (0.6)	2 (0.5)	1 (1.3)	0 (0.0)
P-value*	0.02	0.06	0.06	
OR (95% CI)	17.7 (0.9-344.0)	15.1 (0.7-316.0)	46.0 (1.9-1139.0)	
NOD2 SNP13	461	381	80	1141
-/-	442 (95.9)	365 (95.8)	77 (96.2)	1079 (94.6)
-/C	18 (3.9)	15 (3.9)	3 (3.8)	62 (5.4)
C/C	1 (0.2)	1 (0.3)	0 (0.0)	0 (0.0)
P-value*	0.3	0.2	NA	
OR (95% CI)	7.4 (0.3-183.0)	9.0 (0.4-222)	NA	
CASP1 -8404	469	388	81	1140
AA	281 (59.9)	231 (59.5)	50 (61.7)	650 (57.0)
AG	156 (33.3)	132 (34.0)	24 (29.6)	414 (36.3)
GG	32 (6.8)	25 (6.5)	7 (8.7)	76 (6.7)
P-value*	0.9	1	0.5	
OR (95% CI)	1.0 (0.7-1.6)	1.0 (0.6-1.5)	1.3 (0.6-3.0)	

SNP: single nucleotide polymorphism, BM: bacterial meningitis, MM: meningococcal meningitis, PM: pneumococcal meningitis, OR: Odds Ratio, 95% CI: 95% confidence interval, NA: not applicable
Different numbers in cases are due to different quality of DNA.
P-values and ORs were calculated for homozygous mutant alleles versus WT and heterozygous alleles.
Genotype frequencies of BM survivors were compared to those in controls and MM and PM patients were also separately compared to controls.
* Fisher's exact test.

Table 2. Significant results of carrier trait analyses

SNP combination	Genotype	Total BM n (%)	MM n (%)	PM n (%)	Controls n (%)
TLR4 A>G / TLR2 G>A	GG/AA	13 (2.9)	11 (2.9)	2 (2.5)	4 (0.4)
	All other alleles	440 (97.1)	362 (97.1)	78 (97.5)	1137 (99.6)
	P-value*	3.38x10e-5	4.17x10e-5	0.05	
	OR	8.4	8.6	7.3	
	95% CI	2.7-25.9	2.7-27.3	1.4-40.4	
TLR4 A>G / NOD2 C>T	GG/TT	12 (2.7)	10 (2.7)	2 (2.5)	3 (0.3)
	All other alleles	437 (97.3)	359 (97.3)	78 (97.5)	1138 (99.7)
	P-value*	2.84x10e-5	4.15x10e-5	0.04	
	OR	10.4	10.6	9.7	
	95% CI	2.9-37.1	2.9-38.6	1.6-59.1	

Abbreviations: SNP: single nucleotide polymorphism, BM: bacterial meningitis, MM: meningococcal meningitis, PM: pneumococcal meningitis, OR: Odds ratio, 95% CI: 95% confidence interval.
* Fisher's exact test.

We also found a significant trait with *TLR4* +896 and *NOD2* SNP8. The combination of these SNPs when carrying both homozygous mutant alleles showed a strong association with BM, most pronounced for MM (for BM p=2.8 x 10e-5, OR=10.4, 95% CI=2.9-37.1 and for MM p=4.2 x 10e-5, OR=10.6, 95% CI=2.9-38.6 and not significant for PM).

Other traits with *TLR4* or *NOD2* SNPs did not show a combined effect. We also could not identify significant associations when combining the other SNPs.

DISCUSSION

Comparing genotype frequencies between BM survivors and healthy controls we showed that *TLR4* +896 and *NOD2* SNP 8 were significantly associated with susceptibility to develop MM. The combined carriage of *TLR2* +2477 and *TLR4* +896 mutants as well as the combination of *TLR4* +896 and *NOD2* SNP8 mutants were identified as genetic traits significantly associated with susceptibility to develop MM. Our results were highly statistical significant and were robust after correction for multiple testing. Associations in the PM patient group showed trends in concordance with the results for the MM patients but the number of patients in the pneumococcal cohort was not large enough to be statistical significant for any SNP.

Our study is the first to associate NOD2 with susceptibility to MM, both in single gene as in genetic trait analyses. NOD2 is an intracellular PRR containing a caspase-recruitment domain (CARD) that recognizes bacterial PGN, also present in *N. meningitidis*. *NOD2* SNPs are associated with inflammatory bowel disease and share a signaling defect in response to both the Gram-negative cell wall component

lipopolysaccharide (LPS) as PGN in human experimental studies [22]. Mutant alleles of *NOD2* were associated with decreased activation of NFkB [23]. A genetic susceptibility study in a cohort of pediatric Crohn's disease (Cd) patients showed several genes involved in microbial processing to be associated with Cd development. TLR4 and NOD2 were significantly associated with Cd at the individual level and in gene-gene interactions [24]. *In vitro* studies have shown that NOD2 is expressed by murine microglia and astrocytes and up regulated after exposure to *N. meningitidis* [25, 26]. Experimental studies in murine glial cells have generated hypotheses on the role of these cells in the initiation and progression of inflammation following CNS infection. These experiments have shown that inflammatory responses of both murine astrocytes and microglia are significantly reduced in the absence of NOD2 after stimulation with *N. meningitidis* and that NOD2 plays an important role in astrogliosis, demyelinisation and increased murine locomotor activity after meningococcal infection [14]. These findings are absent in *NOD2* deficient mice. These studies indicate that NOD2 represents an important component in the generation of damaging CNS inflammation following meningococcal infection [14].

The role of TLRs in CNS infection is well recognized and consists of a combination of specific responses to the causative pathogen and also of non-specific activation of the innate immune system [27]. Although synergistic effects for TLR2 and TLR4 have been described for tuberculosis, malaria and lupus, our study is the first to associate a genetic trait for *TLR2* and *TLR4* SNPs with susceptibility to MM [28]. Although TLR2 and TLR4 share the downstream MyD88 pathway resulting in NFkB transcription, it is also known that TLR2 and TLR4 trigger results in differential patterns of gene expression [29]. Toll-interleukin 1-domain-containing adaptor-inducing interferon-β (TRIF) is another class of adapter proteins involved in TLR signaling. TLR4 activation results in the recruitment of both MyD88 and TRIF, whereas TLR2 activation results in the recruitment of MyD88 and not TRIF. MyD88 and TRIF are thought to orchestrate separate gene arms because of temporal differences in how they activate NFkB [29]. Synergy between TLR2 and TLR4 activation has already been described previously in murine macrophages upon stimulation with LPS in the production of TNF-α [30, 31]. Experiments in mice deficient for several redundant PRR showed that TLR2 was the most important receptor to sense Gram-positive cell wall components in a model of pulmonary inflammation. Surprisingly, *TLR4* -/- mice also showed significantly decreased signs of pulmonary inflammation, upon Gram-positive infection indicating a synergistic effect *in vivo* [32].

The combination of *TLR4* and *NOD2* SNPs was also strongly associated with susceptibility to develop MM. At first sight, this combination might not seem very comprehensive considering that TLR4 is a plasma membrane PRR and NOD2 is a cytosolic PRR. Recently, however, the combined effect of these receptors was stud-

ied in a murine macrophage cell line [33]. TLR4 activation with muramyldipeptide increased NOD2 mRNA expression and up regulated NOD2 upon activation. On the other hand, the study proposed that unstimulated NOD2 might play a negative regulatory role in the action of TLR4. The authors concluded that NOD2 has dual effects on TLR4 signaling and exert a novel ligand independent action [33]. Besides, TLR4 and NOD2 were significantly associated with Cd in gene-gene interactions in pediatric cohort of Cd patients [24]. The role of TLR4 and NOD2 was studied in microglia and showed enhanced expression of TLR4 and NOD2 after stimulation with LPS *in vitro* [34].

Carriage of *TLR4* +896 mutants enhances the susceptibility to develop MM in the single gene analysis of our study. TLR4 recognizes LPS in the outer membrane of *N. meningitidis* [35]. TLR4 triggering in combination with LPS binding protein and CD14 leads to an intracellular signaling cascade via MyD88 resulting in the transcription of NFkB and the subsequent production of pro-inflammatory cytokines [36]. *TLR4* +896 mutant alleles are responsible for hyporesponsiveness to LPS in mice and humans in experimental studies [37, 38]. The same *TLR4* SNP has been associated with enhanced susceptibility to Gram-negative infections in adult surgical ICU patients compared to healthy volunteers [39]. In a cohort of children with invasive meningococcal infections *TLR4* +896 was correlated with mortality, increased frequencies of ventilation support, application of inotropic substances, skin grafting, and limb loss [40]. The proposed mechanism in both studies is immune paralysis due to impaired TLR4 mediated LPS responses with decreased pro-inflammatory cell signaling. An experimental study using chimerical *TLR4* mutant mice showed that TLR4 on CNS resident cells such as microglia is critically required for sustained inflammation in the brain after systemic LPS administration independent of systemic cytokines produced upon TLR4 triggering in hematopoietic cells [41].However, in a cohort of 252 Gambian children with serogroup A meningococcal meningitis (of which 120 were culture proven), using a retrospective case-control design, no association was found with *TLR4* +896 and susceptibility to MM [42].

We previously described that *TLR9* +2848 was associated with protection to develop MM. We showed that *N. meningitidis* has a strong immune inhibitory potential upon intracellular recognition by *TLR9*. The *TLR9* +2848 SNP enhances immune responses upon triggering by Neisserial CpG motifs, thereby compensating for the inhibitory potential [11]. We did not involve *TLR9* in our carrier trait analysis since *TLR9* SNPs were associated with protection against MM instead of enhanced susceptibility.

A limitation of this study is the retrospective design. We did not include DNA analysis of the most severe cases of BM in which a child died. However, including the most severe cases probably enhances the associations between SNPs and meningitis susceptibility. Although we used large case and control cohorts, *TLR4* and

NOD2 mutant alleles are rare, as seen by large confidence intervals. This may be due to selection pressure because of the possible adverse effect of these SNPs. Bigger cohorts should be tested and combined with other studies, also in different ethnical populations.

The relevance of identifying genetic variation predisposing for MM development is that it provides better understanding of the details of MM pathogenesis. It also enables the prediction of the individual risk to develop BM and identifies high-risk patients for follow up. In order to be applicable in clinical practice this study has to be confirmed in a second, independent cohort of MM patients. Secondly, this knowledge has to be implemented in clinical practice, for example in existing prediction models and has to be clinically validated. The field of Public Health Genomics is involved in this translation and embraces the Belaggio statement which says that "the responsible and effective translation of genome-based knowledge and technologies into public policy and health services for the benefit of population health" (Bellagio statement, 2005: see www.graphint.org for details) [43, 44].

CONCLUSIONS

In this study we show for the first time that *TLR4* +896 and *NOD2* SNP8 were strongly associated with susceptibility to develop MM in a single SNP analysis. Besides, we identified two genetic carrier traits. Simultaneous carriage of *TLR2* and *TLR4* SNPs and of *TLR4* and *NOD2* SNPs showed an even more pronounced and synergistic association with susceptibility to develop MM. These data may enable the identification of people at risk to develop severe infectious diseases such as MM.

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